OBSERVATIONS ON

THE BACTERIOLOGY OF THE GASTROINTESTINAL TRACT

OF MAN IN HEALTH AND DISEASE

by

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INTRODUCTION

The bacteriology of the gastrointestinal tract of man in health and disease has been a source of interest for several years. Many of the earlier observations were made in patients with pernicious anaemia by investigators outside Britain (Venables and Knott, 1925; Knott, 1927). The great majority of observers at this time believed that in normal fasting subjects the contents of the upper small intestine in the region of the duodenum and upper jejunum were usually sterile on culture, though organisms of oropharyngeal origin might be found. "Not until the middle part of the small intestine is reached do milk souring Gram-positive bacilli become numerous and Gram-negative coliform organisms are not fully established above the lower part of the jejunum" (Knott, 1927). In what was then defined as complete achlorhydria the work of several observers suggested that organisms more usually confined to the colon were present in the small intestine (Bogendörfer, 1922; Gorke, 1922; Venables and Knott, 1925). These studies were extended to patients with pernicious anaemia in an attempt to elucidate the aetiology of this disease and several continental workers (see Knott, 1927) suggested that the bacterial contents of the gastrointestinal tract in pernicious anaemia were very abnormal. Many of these studies were made on the

faeces of patients with pernicious anaemia though some were performed on gastrointestinal aspirates obtained by some method of intubation often only as far as the duodenum. Knott reported the finding of coliform organisms in the duodenal contents of twenty-five of thirty-one patients with pernicious anaemia though the studies do not appear to have been quantitative. Davidson (1928) found that in thirteen of twenty patients with pernicious anaemia the "gastro-duodenal contents" contained significant numbers of coliform organisms and there was considerable support for the theory that organisms such as Escherichia coli or Clostridium welchii produced some toxic factor which brought about this disease. Seyderhelm (1922) went so far as to practise ileostomy and lavage for pernicious anaemia. Useful reviews of the literature of this period are provided by Moench, Kahn and Torrey (1925), Knott (1927) and Davidson and Gulland (1930).

Castle's description of intrinsic factor (Castle and Locke, 1928) rendered the theory of pernicious anaemia as consequent on the absorption of haemotoxic substances obsolescent. The association of an abnormal intestinal bacteriology with macrocytic anaemia had, however, been noted in other situations. Faber (1897) was the first to recognise the association between small intestinal stricture and an anaemia resembling pernicious anaemia and Muelengracht (1921) found that in such a case, the stricture being the result of ileal tuberculosis, the entire small intestine was heavily infiltrated with bacteria. Macrocytic anaemia in association with small intestinal anastomosis was then described by Little, Zerfas and Trusler (1929).

Animal experiments about this period also lent support to the association of abnormal small intestinal anatomy and macrocytic anaemia. Seyderhelm, Lehmann and Wichels (1924) noted macrocytic anaemia in dogs in which they had produced intestinal stenosis and Horster (1935) confirmed these observations. A significant finding was that in the dogs an abnormal and abundant bacterial flora was found throughout the small intestine. Tönnis and Brusis (1931) succeeded in producing similar findings in dogs, this time by forming intestinal cul-de-sacs. Cameron, Watson and Witts (1949b) carried out similar experiments in rats.

Meantime more patients with strictures and abnormal intestinal anastomoses associated with macrocytic anaemia were being recognised and useful reviews are those of Meulengracht (1929), Hurst (1933) and Barker and Hummell (1939). The latter authors found fifty-one cases in the literature, thirty-two of which were due to small intestinal strictures and the rest due to various intestinal anastomoses. Some of the cases had steatorrhoea but macrocytic anaemia was more common. The authors emphasised that they were dealing with a specific disease entity and not with pernicious anaemia and an associated anatomical abnormality of the gastrointestinal tract. Fifty per cent. of the patients they reviewed had free acid in the stomach. They suggested that the features of the condition were related to abnormal bacterial proliferation in the intestinal tract of these patients. Further reviews of similar cases are those of Cameron, Watson and Witts (1949a), Halsted, Lewis and Gasster (1956) and Badenoch (1958).

It was also recognised that jejunal diverticulosis could give rise to a similar syndrome. Taylor (1930) had reported a patient with pernicious anaemia who also had a gastroenterostomy and numerous diverticula in the jejunum, and Montuschi (1949) demonstrated a patient who had steatorrhoea in association with jejunal diverticulosis. Zingg (1950) emphasised that both steatorrhoea and macrocytic anaemia could be associated with jejunal diverticulosis. Badenoch and Bedford (1954) were the first to recognise the triad in a single patient of jejunal diverticulosis, megaloblastic anaemia and steatorrhoea. Though the syndrome is not common many cases have since been described, the most recent review being that of Cooke, Cox, Fone, Meynell and Gaddie (1963).

Quite apart from pernicious anaemia and patients with anatomical abnormalities of the small intestine, several investigators had been interested in the possibility of bacterial infection as a factor in the malabsorptive group of disorders (coeliac disease, idiopathic steatorrhoea and tropical sprue). As early as 1908 Herter suggested that the bacterial flora of the small intestine in coeliac disease was different from the normal and this was supported by the observations of others (Kendall, 1901; Brown, Courtney, Davis and MacLachlan, 1925) which were, however, deduced from the observations on faeces or colonic washings. Manson-Barr (1948) considered also that tropical sprue was infective. Studies on the intestinal contents obtained by aspiration via an intestinal tube in patients with tropical sprue did not, however, lend support to the theory of

abnormal gastrointestinal contamination in this condition (Milanes, Curbelo, Rodriguez, Kouri and Spies, 1946; Nadel and Gardner, 1956) and, in children with coeliac disease and fibrocystic disease, Anderson and Langford (1958) did not find that the gastrointestinal bacteriology differed significantly from that of control subjects. Nonetheless, Frazer (1949) found on intestinal intubation that the intestinal flora was abnormal in the sprue syndrome. Little details of either the method used or the findings were however given. Support for an infective factor in the sprue group of disorders came from observations made on the effects of antibiotics in these conditions. Several observers had noted improvement and even cure in patients with tropical sprue and also idiopathic steatorrhoea following treatment with various antibiotics (French, 1961).

One other situation in which intestinal bacteria were thought to be associated with disease is in the encephalopathy which may occur in liver disease. In these patients the neurological disturbance is associated with the finding of high blood levels of ammonia. This is thought to originate from bacterial action on nitrogenous substances in the gut. The diseased liver is then either unable to detoxicate the ammonia (or other toxic factors) or this substance may bypass the liver via various portosystemic communications. Martini, Phear, Ruebner and Sherlock (1957) had found that in patients with cirrhosis of the liver the small intestinal flora, particularly that of the ileum, differed significantly from that of normal subjects.

THE PRESENT STUDY

The present study, which was initiated in 1961, is the outcome of interest stimulated by Professor R. H. Girdwood in earlier work on the relation of the intestinal bacteriology to certain malabsorptive syndromes (Doig and Girdwood, 1960).

The background to the work has been outlined in the previous section and though there were numerous conditions which were thought to be associated with an abnormal bacterial flora in the gastrointestinal tract of man, there was little objective evidence for this. In patients with intestinal strictures, anastomoses and diverticula for example, little was known of the intestinal bacteriology in these patients. There was much to suggest that this was abnormal, as has already been discussed, and the response of these patients to antibiotics lent support to this theory (Krevans, Lockard-Conley and Sachs, 1954; Badenoch, Bedford and Evans, 1955; Halsted et al., 1956). The numbers of patients in which the flora had actually been studied were, however, very few. Watkinson, Feather, Marson and Dossett (1959) cultured "organisms resembling normal intestinal flora" from the contents of a patient with jejunal diverticulosis. Doig and Girdwood (1960) and Schiffer, Faloon, Chodos and Lozner (1962) grew coliform organisms each from patients with jejunal diverticulosis. All these samples were obtained on the operating table. Lyall and

Parsons (1961) cultured Escherichia coli, Bacteroides and Klebsiella pneumoniae from one of three patients with abnormal small intestinal anastomoses in which they studied the contents of stagnant ileal loop, again obtained at operation, semiquantitatively. It was decided, therefore, to study as many patients with these types of disorders as became available.

Patients who have undergone gastric surgery not uncommonly develop steatorrhoea and may develop megaloblastic anaemia. Many of these patients have a surgically created blind loop and it had been suggested that this might in certain patients give rise to steatorrhoea and anaemia in the same way as occurred in patients with anatomical abnormalities of the small intestine (Bohamsson, 1950; Naish and Capper, 1953; Butler, Capper and Naish, 1954; Adams, 1958). There were no data on the bacteriology of the intestinal tract in these patients and it was therefore resolved to study a group of patients who had undergone such type of surgery.

As far as other malabsorptive disorders are concerned, French (1961) emphasised that previous studies in patients with coeliac disease, idiopathic steatorrhoea and tropical sprue had not excluded the presence of an abnormal gastrointestinal bacteriology. Though Anderson and Langford (1958) did allow their children to take milk during their study, previous investigations had usually been carried out in the fasting state and it might well be that intestinal conditions during normal feeding might be very much more encouraging to bacterial growth. It was therefore also the aim of the study to

investigate a group of such patients.

Soon after these studies were initiated the finding of a normal small bowel flora in a patient with megaloblastic anaemia and a loop in the distal small bowel who in fact had pernicious anaemia, caused the author to review the findings of previous workers in this disease. Moreover, work such as that of Cregan, Dunlop and Hayward (1953) had challenged the importance of gastric secretion in maintaining the gastrointestinal contents free of significant bacterial contamination. The abnormal findings previously described in pernicious anaemia were therefore intriguing and no work had been done on the subject since Davidson had described his findings in 1928. With the introduction of the concept of deficiency of intrinsic factor as the cause of pernicious anaemia (Castle and Locke, 1928) interest in the gastrointestinal bacteriology of these patients, which had been stimulated essentially because of the possible causative role of an abnormal bacteriology in the condition, was lost. Since then many other causes of megaloblastic anaemia had been defined and paradoxically there appeared to be good evidence that at least in some of these, as had been once thought of pernicious anaemia, an abnormal gastrointestinal flora was of aetiological significance. It was possible therefore that some of the previously studied cases of pernicious anaemia included other disorders now also known to cause megaloblastic anaemia. The much less stringent criteria for gastric anacidity also made this possible. It was therefore decided to study a group of patients with pernicious anaemia. This interest has subsequently

spread to the study of the gastric intrinsic factor level in patients with pernicious anaemia, in relation to the bacteriological findings in the gastrointestinal tract, as will be discussed later.

In summary, therefore, the aim of the present work was the study of the bacteriology of the small intestine in various malabsorptive disorders including patients with anatomical abnormalities of the gastrointestinal tract. For the reasons already made it was desirable that patients should not be fasting throughout the investigation. A group of patients with pernicious anaemia would also be studied. Because the literature includes only one study of patients with cirrhosis it was also decided to investigate such patients as they became available. The bacteriological findings would be interpreted in the light of the findings in a group of control subjects. It is appropriate to emphasise at this point that all the subjects were volunteers in that it was explained to them that the investigation was not necessary for their management.

Finally, though with greater knowledge of the bacteriological content of the small intestine in certain conditions has come the general acceptance that in certain situations it may be the cause of megaloblastic anaemia and of steatorrhoea, there is little understanding of how these come about. The present work describes some studies <u>in vitro</u> with some organisms isolated from the gastrointestinal tract in an attempt to elucidate the method by which they might bring about deficiency of vitamin B_{12}.

MATERIALS AND METHODS*

1. Subjects studied: A total of 152 subjects have been studied. They are divided into the following groups.

(i) Twenty-six control subjects and ten others

These subjects did not suffer from any known disorder of the gastrointestinal tract. A number of these subjects were studied during a visit to the Medical College, Baroda, India, in 1965. In addition to these there were another ten subjects with miscellaneous disorders. Three of these were taking corticosteroid therapy, two chemotherapeutic agents and one was studied during an attack of mild gastroenteritis though no pathogenic organisms were isolated on stool culture. Four of these subjects were also Indians studied during the author's visit to the Medical College, Baroda, and were taking propantheline bromide as volunteers.

(ii) Eight patients with primary malabsorptive disease.

The diagnosis was made on the basis of studies of small intestinal function and histological examination of the jejunal mucosa. The stool fat content was determined over a period of 5 - 18 days by the method of van de Kamer (1949), an output of

*See also Appendix I

greater than 7 gm. daily being considered to be abnormal. The absorption of carbohydrate was measured by measuring the excretion of xylose in the urine in the 5 hr after an oral dose of 25 gm. Normal subjects excrete more than 4 gm. The serum vitamin B_{12} level was determined microbiologically using Lactobacillus leichmannii as the test organism. Our normal range is 170 - 1000 uugm./ml. Vitamin B_{12} absorption was measured using the urinary excretion method (Schilling, 1953). The serum folate was measured microbiologically using Lactobacillus casei as the test organism. Our normal range is 4.9 - 18.5 mugm./ml. (Kershaw and Girdwood, 1964). The excretion of formiminoglutamic acid (Figlu) was measured using conventional voltage electrophoresis (Kohn, Mollin and Rosenbach, 1961; Kershaw and Girdwood, 1964). Folic acid absorption was determined microbiologically using the urinary excretion method of Girdwood (1956) with Streptococcus faecalis as the test organism. The author performed the jejunal biopsies using the Crosby-Kugler capsule (1957), the position of which was confirmed radiologically prior to the biopsy being taken. The criteria for normality have been discussed elsewhere (Girdwood, Williams, McManus, Dellipiani, Delamore and Kershaw, 1966). All the patients routinely underwent radiological examination of the small intestine and measurement of gastric acid secretion after maximal histamine stimulation (Kay, 1953; Card, Marks and Sircus, 1955). Two had histamine-fast achlorhydria.

All these patients had impaired absorption of folic acid and histological examination of the jejunal mucosa revealed either sub-

total or total villous atrophy on light microscopy and ridges or more advanced changes on dissecting microscopy. Two patients were on a gluten-free diet and one on steroid therapy at the time of the study, the others being untreated.

(iii) Twenty patient with pernicious anaemia.

The diagnosis of pernicious anaemia was made on the finding of megaloblastic anaemia associated with a low serum vitamin B_{12} level, achlorhydria on maximal histamine stimulation (Kay, 1953; Card, Marks and Sircus, 1955) and an abnormal Schilling test which was correctable by intrinsic factor. All patients responded to treatment with perenteral vitamin B_{12} and most of the patients had been treated at the time of study. In all the patients the small bowel was examined radiologically and in one a small intestinal diverticulum was present.

(iv) Four patients with low acid secretion.

Three of these had histamine-fast achlorhydria and one 0.9 m.Eq. hydrochloric acid in the post-histamine hour.

(v) Twenty-two patients with blind or stagnant loops.

Ten of these patients had diverticula in the small

intestine demonstrated radiologically and in nine these were multiple. The other twelve patients had acquired their loops surgically except in one case in which an intestinal fistula had occurred spontaneously due to Crohn's disease. Of the patients with surgical loops nine had had an ileo-transverse colostomy, one had had an ileal resection with an end-to-side anastomosis and in another patient the exact site of the loop could not be determined.

In all these patients the serum vitamin B_{12} level, measurement of vitamin B_{12} absorption and steatorrhoea were measured as already described. In addition to measuring vitamin B_{12} absorption after intrinsic factor, this was also done on the 4th day of a five day course of Tetracycline, 250 mg. four times daily, in those in whom vitamin B_{12} absorption was impaired. The incidence of steatorrhoea in these patients was low. Two patients with jejunal diverticula had steatorrhoea.

It should be pointed out that these are two different groups of patients. Thus with the exception of three patients (87, 88 and 89 in Table 10) all the subjects with loops in the distal small bowel had presented with symptoms of tiredness or anaemia and had impaired absorption of vitamin B_{12}. In contrast, in the majority of patients with jejunal diverticula these had often been found incidentally during the investigation of these patients for some other complaint.

The data on the gastric acid secretion of these patients are somewhat incomplete. Some, particularly those with loops in the distal small bowel, were studied before the maximal histamine test

was available, some refused another intubation procedure for what had been explained to them were research purposes and, in the patient studied in India facilities for the study of gastric secretion were not available. The results of acid secretion studies in the patients with diverticula will be indicated in the appropriate table. Of the other patients all were known to have free acid present in the stomach except for four. One had histamine-fast achlorhydria and was in fact a case of pernicious anaemia. In the other three patients vitamin B_{12} absorption was normal and gastric acid secretion was not studied.

(vi) Forty-seven patients with gastric surgery.

These consisted of twenty-six patients who had undergone partial gastrectomy with a Billroth II type anastomosis, and twenty-one who had undergone gastroenterostomy and vagotomy. In every case surgery had been carried out as part of the management of duodenal ulceration. The mean interval since operation with standard deviation was 11.3 ± 3.6 years after partial gastrectomy and 4.4 ± 1.7 years after gastroenterostomy and vagotomy.

In these patients, steatorrhoea, the serum vitamin B_{12} level, the Schilling test and the acid secretion of the gastric remnant were measured as has already been described. No attempt was made to block off the gastroenterostomy stoma during the measurement of acid secretion. An attempt was made to assess the incidence of diarrhoea

objectively. Thus a patient was said to be suffering from diarrhoea if he or she passed at least four bowel motions daily and had not done so prior to operation. Some patients suffered from intermittent diarrhoea.

(vii) Fifteen patients with disorders of the liver.

Twelve patients had cirrhosis of the liver and six of this group were studied during a visit to the Medical College, Baroda, India. The clinical status of these patients was very variable. In most of these patients the diagnosis was straightforward from the history, clinical and investigative findings and from the subsequent progress. When in doubt the diagnosis was confirmed by liver biopsy using a Menghini needle (1958) carried out by the author. In the light of the findings on liver biopsy a presumptive diagnosis of cirrhosis in two patients was reviewed to one of recurrent hepatitis.

The study of gastric secretion in these patients is incomplete. This is largely because in the six patients studied in India facilities for such studies were not available. Three of the patients with alcoholic cirrhosis refused a further intubation procedure. One patient with cirrhosis was known to have histamine-fast achlorhydria.

2. Method of Sampling

(i) Methods of sampling at jejunal and ileal level.

The method used was a nasal intubation technique. The tube was similar to that suggested by Blankenhorn, Hirsch and Ahrens (1955) who carried out transintestinal intubation in various subjects for metabolic studies. It was of such a nature as to withstand autoclaving, expose the patient to the minimum of discomfort and yet allow adequate aspiration. Six foot lengths of polyvinyl tubing (Grade: E.R.P., Esco (Rubber) Ltd., London) were used. The bore of the tube was 1.5 mm. and the external diameter 2.5 mm. Aspirating ports were cut in the region of the terminal three to four inches and a mercury bag (containing 1.5 - 2 ml. mercury) attached to the end. The bags were made up using ordinary ward finger cots. To prevent stagnation of aspirated contents in the tube, the region between the distal aspirating port and the mercury bag was closed off by obliterating the bore of the tube just distal to this port with strong thread. Radioopaque markers were attached to the tube in the form of $\frac{1}{2}$ inch lengths of the radioopaque thread used in surgical swabs. These were attached with a small piece of zinc oxide plaster (Fig. 1).

All materials were autoclaved prior to intubation of the fasting patient in the morning when a fine plexitron tube was introduced nasally. The end of this was drawn out of the patient's mouth and to it the proximal end of the polyvinyl tube was attached with a

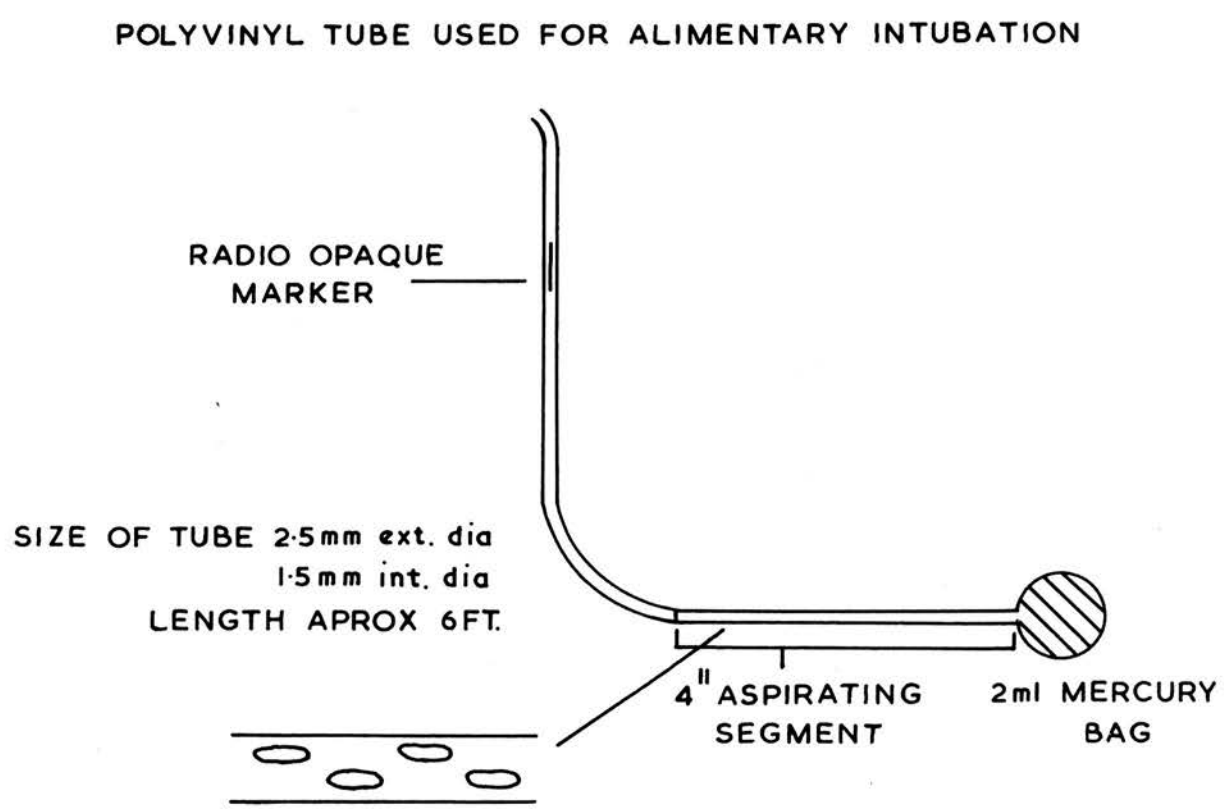


Figure 1. Polyvinyl tube used in study.

sterile metal segment. Thus the polyvinyl tube could be drawn back out of the patient's nose, this being continued until, when the distal end with the mercury bag entered the mouth, the patient was allowed to swallow this. The proximal end of the polyvinyl tube, i.e. the site of attachment to the plexitron tube, was regarded as contaminated and cut off. Further lengths of polyvinyl tubing were attached by sterile metal segments as and when required.

The tube was washed through using a sterile syringe and sterile needle connection with 100 ml. of sterile saline after it had reached the stomach and this was repeated after the aspirating segment had negotiated the pylorus and again on the completion of the jejunal aspiration. After the tube was washed through air was blown through and to prevent the entry of intestinal contents into the tube the proximal end was sealed. Aspiration was carried out when the aspirating ports were calculated and assessed by fluoroscopy to be situated one to two feet beyond the duodeno-jejunal junction. The initial volume of aspirate was rejected. A similar procedure was carried out at mid-ileal level, i.e. after the tube had passed 4 - 5 feet beyond the site of jejunal aspiration. Some delay in aspiration occurred here owing to the various times, often at night, at which the tube reached the desired site. There was always an interval of at least four hours between the time the tube had previously been washed through with 100 ml. of sterile saline and the time of aspiration at jejunal or ileal level.

(ii) Method of sampling at gastric and jejunal level.

Most patients were studied in this manner. The duration of intubation in these cases was shorter and with greater experience (including personal experience) it became apparent that oral intubation put the patients to little discomfort and appeared to be preferable to the nasal route. Thus these patients were intubated orally and fasting gastric juice was aspirated. Aspiration of gastric juice was not always successful particularly in patients with low acid secretion. The procedure was subsequently as has already been described. In patients with a partial gastrectomy or a gastroenterostomy, however, the tube was not washed through again after it had entered the small intestine. In all these patients the studies were complete by late afternoon.

In all patients, passage through the pylorus was aided by turning them initially on the right side. They were allowed a normal ward diet throughout the experimental period starting immediately after intubation in those undergoing jejunal and ileal aspiration, and immediately after the taking of the fasting gastric aspirate in those undergoing gastric and jejunal aspiration. In general, all patients were studied if possible whilst they were ambulant. Indeed most of the patients with pernicious anaemia were studied as out-patients. In certain cases this was not possible because of the patient's

general condition. Thus some of the patients with cirrhosis of the liver and two of the patients on corticosteroid therapy for leukaemia had been confined to bed for several days prior to the period of study.

3. Bacteriological study of the aspirates

The aspirates obtained from the gastrointestinal tract were made up into serial dilutions ($\frac{1}{10}$ to $\frac{1}{10^5}$) using nutrient broth. The gastric juice was sometimes somewhat viscid and dilutions were therefore made up in screw cap universal containers which permitted shaking. Viable counts were done on each specimen using the technique of Miles and Misra of serial dilution with drop counts (1938). The method would demonstrate organisms in as low a concentration as 250 per ml. All estimations were performed in duplicate. In those patients undergoing jejunal and ileal aspiration it was hoped to isolate as many organisms as possible by using selective as well as ordinary media. Thus blood agar and MacConkey agar plates were cultured aerobically whilst blood agar, Willis and Hobbs' medium (1959) (for clostridia), Rogosa agar (Oxoid modification P.M.221 of the formula of Rogosa, Mitchell and Wiseman, 1951) (for lactobacilli),

neomycin blood agar (Smith and Crabb, 1961) (for Bacteroides) and thallus acetate agar plates (Barnes, 1956) (for anaerobic streptococci) were cultured anaerobically. The characteristics of individual colonies were noted and examined by Gram staining. Clostridium welchii were identified by the presence of opalescence (lecithinase production) and lactose fermentation around the colony.

In those patients undergoing gastric and jejunal aspiration, organisms of the faecal type as represented by Enterobacteriaceae and Streptococcus faecalis were searched for on MacConkey and blood agar. In addition Bacteroides and lactobacilli were looked for as described above. Enterobacteriaceae were subdivided by their biochemical reactions to confirm closely with the groups proposed by the Enterobacteriaceae Subcommittee of the International Committee on Bacterial Nomenclature and Taxonomy (Enterobacteriaceae Subcommittee, 1958) (Table 1).

Because of limited facilities in the patients studied in India only Enterobacteriaceae and Streptococcus faecalis were looked for. Bacteroides are difficult organisms to grow and the neomycin blood agar plates were therefore cultured anaerobically for five days. These organisms are, however, said to grow better under microaerophilic conditions and thus during the latter part of the study carbon dioxide (10%) was added to the jars in which these organisms were grown.

TABLE 1

Family	Genera
Enterobacteriaceae	Escherichia
	Alkalescens-Dispar
	Citrobacter
	Klebsiella
	Cloaca
	Hafnia
	Proteus
	Providencia
	Salmonella
	Arizona
	Shigella

4. Studies in vitro - the uptake of vitamin B_{12} by microorganisms.

In an attempt to elucidate what part organisms isolated from these patients might be playing in relation to vitamin B_{12} absorption, the uptake of radioisotopically labelled vitamin B_{12} by these organisms was studied *in vitro*.

The ability of organisms to assimilate cyanocobalamin was estimated by preparing 10 ml. aliquots of a mixture of 150 ml. of Difco microinoculum broth (pH 6.8) to which 1 ug. ^{58}Cobalt-labelled cyanocobalamin (Radiochemical Centre, Amersham) had been added. This is approximately twelve times the amount of vitamin B_{12} in a similar volume of Difco microinoculum broth. The solution was sterilised at 15 lb. pressure for 10 minutes. One ml. of an overnight (sixteen hour culture) of an organism was added to an aliquot. Two standards of 10 ml. of broth with added ^{58}Cobalt-labelled cyanocobalamin, but inoculated with 1 ml. sterile saline, were used as controls. After overnight incubation at 37° C the material was centrifuged at 3,000 revolutions per minute for 30 minutes. The extent of the radioactivity in the supernatant was calculated using a well type scintillation counter and compared with that in the uninoculated control samples. Measurement of the radioactivity in the organisms confirmed that they had removed it from the culture medium. The radioactivity could not be washed off the organisms.

The rate of uptake of cyanocobalamin was investigated by

preparing 6 x 10 ml. of broth containing the ^{58}Cobalt-labelled cyanocobalamin, prepared as described above, and adding to this one ml. of a fresh overnight culture of the organism under study. Centrifugation was done as above after incubation at zero time, one hour, two hours, five hours, 12 hours and 24 hours, and the activity of the supernatant calculated at each time.

The experience derived from this *in vitro* work resulted in the planning of other experiments, the object of which was the study in more detail of factors affecting the uptake of vitamin B_{12} by *Escherichia coli*, the commonest organism we have isolated from the gastrointestinal tract. All these experiments were carried out on a single strain of *Escherichia coli*, an organism isolated from the gastrointestinal tract of a patient with a gastroenterostomy. There was considerable variability in the details of this work and thus the individual details and results are included in the Appendix.

In general, cultures of *Escherichia coli* were prepared by inoculating one ml. of an overnight culture into Difco microinoculum broth which was then incubated at 37° C. The cultures were usually set up in 10 ml. of broth, though in some experiments other volumes were used. The object was to study the effect of growing cultures and they were therefore used exactly four hours after initial inoculation from the overnight culture. Occasionally older cultures were used

as described. Labelled cyanocobalamin was added to the cultures, according to the conditions of the experiment, as 1/15 ugm. of the ^{58}Cobalt-labelled vitamin in 0.1 ml. water. The uptake of the labelled vitamin by the organisms was determined after a further period of incubation of one hour though in some experiments the period of incubation was extended as will be described. The uptake was calculated by comparing the radioactivity of the supernatant after centrifugation with that of uninoculated standards as described above.

By combining measured volumes of gastric juice with the cultures the uptake of vitamin B_{12} by the organisms in the presence of the juice was determined. Human gastric juice was obtained from subjects after augmented histamine stimulation and the pepsin in this inactivated by alkaline denaturation as described by Grässbeck (1960) except that buffer was not added to prevent dilution of binding factors. The juice was collected in 20 minute aliquots under ice. The pH was taken to 10 with normal sodium hydroxide and kept at this value for twenty minutes. The pH was then brought to 7 using normal hydrochloric acid. It was then kept at -20° C until required. In the earlier part of the work juice was sterilised by filtration through standard Berkefeld filters (British Berkefeld Filters Ltd., No. 8). The results of work using such juice were such that this form of preparation had to be abandoned.* Later experience in fact showed that juice could be used without subjecting it to sterilising

*see Appendix II

procedures. Human gastric juice with an output of more than 20 m.Eq. acid in the post-histamine hour or of known intrinsic factor concentration was used. The latter was determined by the immunoassay method of Ardeman and Chanarin (1963).

The effect of gastric juice which had been exposed to enzyme digestion on the uptake of vitamin B_{12} by the microorganisms was also studied. For this work crystalline pepsin, trypsin and chymotrypsin were employed (Worthington, U.S.A.) in a concentration of 2.5 mg. (in 0.1 ml. of water) per ml. of gastric juice. This enzyme concentration is based on observations made on their concentration in human gastric and pancreatic juice (Babkin, 1950; Lagerlöf, 1942; Reisenstein, 1959). Enzyme digestion was carried out for four hours at pH2 with pepsin and at pH8 with trypsin and chymotrypsin. Following the period of digestion with pepsin this enzyme was inactivated by alkaline denaturation. Inactivation of trypsin and chymotrypsin was not carried out. This was because it was thought that chemical contamination of the media with enzyme inactivators was undesirable. Again the pH adjustments required to produce inactivation of these enzymes were such that they would have resulted in the destruction of intrinsic factor. The fact that incubation after the exposure to the four hour digestive period with the enzymes was for one hour only and that, as it turned out, the effect of trypsin and chymotrypsin on the binding activity of gastric juice was very small indicates that any error involved due to failure to inactivate trypsin and chymotrypsin is likely to be insignificant.

Throughout all this *in vitro* work appropriate controls were set up. Particular care was taken to detect bacterial contamination of the various solutions employed. If this occurred the experiment was rejected.

RESULTS

Control subjects

In Table 2 are shown the findings obtained on culturing the jejunal and ileal aspirates for the organisms previously mentioned in six hospital control subjects. The thyrotoxic patient was mildly toxic. In Table 3 are illustrated the findings in a further series of control subjects who had their gastric and jejunal aspirates cultured for faecal type organisms only. Table 4 shows the findings in a series of Indian subjects, the condition of the patients and the dietary habits being as illustrated. In these twenty-six subjects it was most unusual to find faecal type organisms in the upper small intestine. Lactobacilli were also unusual. In two of the six patients in whom the ileal contents were studied faecal type organisms were present. As far back as 1919 Bessau and Bossert stated that they regarded as abnormal the presence of even a few coliform bacilli in the duodenal juice. The author would agree with this and would classify a concentration of faecal type organisms, as represented by the family Enterobacteriaceae and Streptococcus faecalis, of 10^4/ml. as abnormal in the upper small intestine. In view of the findings

TABLE 2

Bacteriological findings in the jejunum and ileum of six control subjects

Case No.	Organisms found	Jejunal Aspirate	Ileal Aspirate	Diagnosis
1	Strep. viridans Staph. albus Cl. welchii	3×10^5 1×10^6 -	- - 1×10^4	Normal
2	Lactobacilli	-	5×10^3	Hysterical neurosis
3	-	-	-	Rheumatoid arthritis
4	Strep. viridans Staph. albus	2×10^5 1×10^6	- -	Thyrotoxicosis
5	Diphtheroid bacilli Yeasts	7×10^4 -	1×10^4 1×10^4	Hypochondriasis
6	Strep. viridans Escherichia	5×10^4 -	- 4×10^6	Obesity

Results expressed as numbers of viable organisms per ml. aspirate

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

TABLE 3

Faecal type flora in stomach and jejunum of nine control subjects

Case No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Diagnosis
7	Strep. faecalis	2×10^3	-	Epilepsy
8	-	-	-	Thyrotoxicosis
9	-	-	-	Allergy
10	-	-	-	Testicular seminoma
11	-	-	-	" "
12	-	-	-	" "
13	Citrobacter	-	5×10^2	" "
14	Strep. faecalis Lactobacilli	5×10^3 -	- 3×10^2	Normal
15	-	-	-	Normal

Results expressed as numbers of viable organisms per ml. aspirate

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

TABLE 4

Faecal type flora in stomach and jejunum of eleven control subjects studied at The Medical College, Baroda, India

Case No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Diagnosis	Diet
16	-	-	-	Doctor	Vegetarian
17	-	-	-	Technician	Vegetarian
18	Citrobacter	-	2×10^3	Student	Mixed
19	-	-	-	Student	Mixed
20	-	-	-	Student	Mixed
21	-	-	-	Student	Mixed
22	-	-	-	Thyrotoxicosis	Vegetarian
23	-	-	-	Muscular dystrophy	Vegetarian
24	-	-	-	Pharyngitis	Vegetarian
25	Escherichia	-	8×10^3	No diagnosis	Vegetarian
26	-	-	-	Mitral stenosis	Vegetarian

Results expressed as numbers of viable organisms per ml. aspirate

In this group of patients lactobacilli and Bacteroides were not looked for

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

in the stomach this definition should apply to that organ also. It is likely that a similar definition could be applied to lactobacilli and the findings in these patients would suggest that the mere presence of Bacteroides would be abnormal.

In Table 5 are the findings in ten further subjects, one of which had had mild gastroenteritis whereas the others were receiving numerous drugs. Patients 30 and 31 were receiving 60 mg. Prednisone daily as treatment for acute leukaemia. Both were very toxic. Patient 32 was receiving 15 mg. Prednisone daily. Patients 33 - 36 were receiving propantheline bromide, 15 mg. four times daily. The patients with the gastrointestinal upset, those on chemotherapy and two of the patients on steroid therapy exhibited abnormal bacteriological findings.

Patients with malabsorptive disease

Table 6 shows the findings of full bacteriological investigation in the jejunum and ileum of eight patients with primary malabsorptive disease. Two patients, Nos 39 and 41, had histamine-fast achlorhydria. Patients Nos 37 and 43 were on a gluten-free diet and patient 39 was on corticosteroid therapy (Prednisone, 60 mg. daily).

TABLE 5

Faecal type flora in stomach and jejunum of patients with miscellaneous disorders

Case No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Comments
27	Klebsiella Citrobacter Lactobacilli	Not done Not done Not done	5×10^4 1×10^6 4×10^5	"Infective" diarrhoea No pathogens isolated
28	Strep. faecalis Escherichia	1×10^3 -	- 1×10^5	On sulphonamides Renal colic
29	Klebsiella Alk. Dispar Bacteroides Lactobacilli	Not done Not done Not done Not done	2×10^6 3×10^3 2×10^3 2×10^5	On carbimazole On nitrofurantoin Urinary tract infection
30	Escherichia Strep. faecalis	3×10^4 2×10^3	2×10^6 2×10^4	On steroid therapy Leukaemia
31	Klebsiella Alk. Dispar	- -	4×10^3 1×10^4	On steroid therapy Leukaemia
32	Escherichia Lactobacilli	7×10^3 4×10^4	- -	On steroid therapy Idiopathic thrombocytopaenic purpura
*33	-	-	-	Indian student On propantheline bromide
*34	Escherichia	-	3×10^3	Indian student On propantheline bromide
*35	-	-	-	Indian student On propantheline bromide
*36	Escherichia	3×10^3	-	Indian patient Pneumothorax On propantheline bromide

Results expressed as numbers of viable organisms per ml. aspirate

*Lactobacilli and Bacteroides not looked for

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

TABLE 6

Bacteriological findings in the jejunum and ileum of eight patients with malabsorptive disease

Case No.	Organisms found	Jejunal Aspirate	Ileal Aspirate
37	Lactobacilli	-	4×10^4
	Escherichia	-	2×10^4
	Alk. Dispar	-	2×10^7
	Yeasts	4×10^2	1×10^6
	Anaerobic streptococci	-	4×10^5
38	Lactobacilli	-	4×10^3
	Escherichia	-	1×10^7
	Yeasts	2×10^3	1×10^4
39	Lactobacilli	1×10^4	4×10^4
	Strep. faecalis	4×10^5	3×10^5
	Escherichia	4×10^4	3×10^6
	Cl. welchii	-	2×10^3
40	Strep. viridens	6×10^5	1×10^6
	Staph. albus	3×10^5	-
	Neisseria	2×10^3	-
	Lactobacilli	1×10^3	2×10^3
	Strep. faecalis	3×10^5	2×10^7
	Citrobacter	2×10^3	4×10^6
41	Strep. viridans	2×10^4	-
	Neisseria	1×10^4	-
	Lactobacilli	-	3×10^4
	Cloaca	-	1×10^8
	Yeasts	-	6×10^5
42	Strep. viridans	3×10^3	1×10^3
	Yeasts	3×10^3	5×10^2
43	Strep. viridans	7×10^3	4×10^2
	Lactobacilli	3×10^3	1×10^3
	Yeasts	4×10^3	4×10^4
44	Lactobacilli	1×10^4	4×10^3
	Yeasts	4×10^2	1×10^3

Results expressed as numbers of viable organisms per ml. aspirate

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

This last patient died of uncontrolled malabsorptive disease some days after the examination. As is seen, faecal type organisms were found in abnormal numbers in patients Nos 39 and 40, in the latter only the concentration of Streptococcus faecalis being significant. Yeast type organisms and lactobacilli were generally found only in small concentrations in the upper small bowel. Patients Nos 39 and 40 had quite a high concentration of Streptococcus faecalis and patients Nos 37, 38, 39, 40 and 41 of coliform organisms in the ileum. Lactobacilli and yeasts occurred, usually in low concentrations, in the ileum of most patients.

Patients with pernicious anaemia and low acid secretion

The results of examining the gastric and jejunal aspirates in these patients for faecal type organisms are illustrated in Table 7. The first twenty patients (Nos 45 - 64) had pernicious anaemia. Patients Nos 65, 66 and 67 had histamine-fast achlorhydria and patient 68 had an output of only 0.9 m.Eq. of hydrochloric acid in the post-histamine hour. Of the patients with pernicious anaemia Nos 51, 55, 56, 58, 59 and 61 had abnormal numbers of faecal type organisms in the jejunum. Patient No. 55 had a duodenal diverticulum.

TABLE 7

Faecal type flora in stomach and jejunum of twenty patients with Pernicious anaemia and four patients with low acid secretion

Case No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	
45	-	Not done	-	Pernicious Anaemia
46	Lactobacilli	Not done	5×10^2	" "
47	-	Not done	-	" "
48	-	Not done	-	" "
49	-	-	-	" "
50	-	-	-	" "
51	Escherichia	1×10^8	1×10^8	" "
52	-	-	-	" "
53	-	-	-	" "
54	-	-	-	" "
55	Escherichia Proteus	7×10^6 -	3×10^6 1×10^7	" " " "
56	Escherichia Strep. faecalis	- -	4×10^3 2×10^6	" " " "
57	Strep. faecalis	1×10^3	5×10^3	" "
58	Strep. faecalis	-	2×10^5	" "
59	Alk. Dispar	4×10^6	2×10^7	" "
60	-	-	-	" "
61	Escherichia	-	1×10^4	" "
62	-	-	-	" "
63	Citrobacter Lactobacilli	4×10^3 4×10^3	- 1×10^3	" " " "
64	Lactobacilli	5×10^4	3×10^6	" "
65	Lactobacilli	5×10^3	-	Achlorhydria
66	Escherichia Lactobacilli	2×10^3 7×10^5	2×10^4 7×10^3	" "
67	Escherichia Lactobacilli	2×10^4 2×10^6	2×10^3 2×10^5	"
68	Strep. faecalis	5×10^7	2×10^8	0.9 m.Eq. acid

Results expressed as numbers of viable organisms per ml. aspirate
- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

Two patients with low gastric acid secretion had abnormal numbers of coliform organisms and enterococci in the jejunum and in the stomach.

Table 8 shows the results of more extensive bacteriological studies at jejunal and ileal levels in five of these patients (Nos 45 - 48). It will be seen that Clostridium welchii were found in the ileum in two patients. In general, therefore, about one-third of this whole group exhibited abnormal findings.

Patients with blind or stagnant loops

Table 9 illustrates the bacteriological findings of faecal type flora in ten patients with jejunal diverticula. Patient No. 77 had had a partial gastrectomy as well. Patient No. 78, who was studied in India, had a single diverticulum present only. Four patients, Nos 69, 70, 73 and 77, had abnormal absorption of vitamin B_{12} which in the first three patients was corrected by antibiotics. Only two patients, Nos 73 and 77, had steatorrhoea. In this group of patients as a whole seven of the ten patients had an abnormal flora and this included the patient with a single jejunal diverticulum. Bacteroides were found in one patient (No. 76). It will be noted that an abnormal flora was found in some patients in the presence of gastric

TABLE 8

Bacteriological findings in the jejunum and ileum of five patients with pernicious anaemia

Case No.	Organisms found	Jejunal Aspirate	Ileal Aspirate
45	Strep. viridans	1×10^6	1×10^4
46	Strep. viridans	2×10^6	4×10^4
	Staph. albus	2×10^6	5×10^6
	Lactobacilli	5×10^2	4×10^3
47	Strep. viridans	2×10^6	-
	Staph. albus	1×10^6	-
	Cl. welchii	-	1×10^4
48	-	-	-
49	Strep. viridans	2×10^6	5×10^5
	Staph. albus	3×10^3	-
	Lactobacilli	-	4×10^4
	Coliform bacilli	-	6×10^6
	Cl. welchii	-	5×10^5

Results expressed as numbers of viable organisms per ml. aspirate

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

TABLE 9.

Faecal type flora in stomach and jejunum of ten patients with jejunal diverticula

Case No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Vit.B_{12} Absorption	Presentation	Acid Secretion mEq.
69	Klebsiella (3)	-	1×10^7	Abnormal (1)	Iron Defeciency Anaemia	Present
70	Escherichia (3)	Not Done	5×10^8	Abnormal (1)	Gastrointestinal Symptoms Megaloblastic Anaemia	Present
71	Escherichia (3) Strep. faecalis	4×10^3 8×10^3	5×10^2 -	Normal	Gastrointestinal Symptoms	Not Done
72	Escherichia Lactobacilli	- -	1×10^7 7×10^4	Normal	Gastrointestinal Symptoms	Not Done
73	Klebsiella Lactobacilli	Not Done	5×10^6 1×10^5	Abnormal (1)	Megaloblastic Anaemia Steatorrhoea (10 G daily)	1.6
74	-	-	-	Normal	Gastrointestinal Symptoms	13.0
75	-	-	-	Normal	Sideroblastic Anaemia	1.0
76	Alk. Dispar Klebsiella Strep. faecalis Lactobacilli	- - - 5×10^5	5×10^6 5×10^5 5×10^4 9×10^3	Normal	Gastrointestinal Symptoms	6.0
77	Escherichia Strep. faecalis Bacteroides Lactobacilli	2×10^3 - - 2×10^6	8×10^6 1×10^6 5×10^5 2×10^4	Abnormal (2)	Partial Gastrectomy Steatorrhoea (8.5 G daily)	0.1
78	Escherichia (3) (4)	6×10^3	8×10^7	Not Done	Parkinson's Disease	Present

(1) Absorption corrected by antibiotics

(2) Absorption corrected by intrinsic factor

(3) Lactobacilli and Bacteroides not looked for

(4) Single diverticulum present

- in columns 2, 3 and 4 indicate organisms present in concentration of less than 250 per ml. aspirate -

Results expressed as numbers of viable organisms per ml. aspirate

acidity but in those in which this was measured acid output was low.

In Table 10 are the findings in twelve patients with loops (mainly ileo-transverse colostomies) involving the small bowel. None of these patients had steatorrhoea. Only patients Nos 88, 89 and 90 had normal absorption of vitamin B_{12}. Antibiotics improved vitamin B_{12} absorption in four patients and in one, No. 87, it was corrected by intrinsic factor. Of the patients with impaired vitamin B_{12} absorption significantly affected by antibiotic therapy, three had an abnormal flora in the jejunum and one in the stomach. Of the other patients one, No. 83, had abnormal jejunal findings. This patient had subacute intestinal obstruction associated with a colonic tumour.

Patients with gastric surgery

In Table 11 are found the bacteriological studies in twenty-six patients who had undergone partial gastrectomy, and in Table 12 those in twenty-one who had undergone gastroenterostomy with vagotomy.

Faecal type organisms were often cultured from the gastric and jejunal juices of these patients, the findings being similar regardless of the previous type of surgery. In about one-third of the

TABLE 10

Faecal type flora in stomach and jejunum of twelve patients with loops in the lower small bowel

Case No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Vit.B_{12} Absorpt. after Tetracycline	Diagnosis
79	Escherichia	3×10^4	-	Improved	Ileo-transverse colostomy for appendicitis
80	Strep. faecalis Escherichia Klebsiella Proteus	2×10^3 - 1×10^3 -	1×10^3 3×10^3 - 2×10^4	Corrected	Ileo-transverse colostomy for appendicitis
81	Escherichia	-	2×10^7	Corrected	Ileo-transverse colostomy for appendicitis
82	Escherichia Lactobacilli	Not done	4×10^6 4×10^4	Corrected	Ileo-transverse colostomy for appendicitis
83	Klebsiella	Not done	5×10^6	Abnormal	Ileo-transverse colostomy for Crohn's disease.Colonic cancer
84	-	-	-	Abnormal	Ileo-transverse colostomy for Crohn's disease
85	-	-	-	Abnormal	Ileo-transverse colostomy for appendicitis
86	Strep. faecalis	6×10^3	-	Abnormal	Ileo-transverse colostomy for adhesions
87	-	-	-	Abnormal	Ileal resection Pernicious anaemia
88	Escherichia	2×10^3	2×10^3	Not done	Ileo-transverse colostomy for Crohn's disease.Gastro-enterostomy
89	Alk. Dispar Strep. faecalis Lactobacilli	3×10^3 2×10^3 3×10^4	- -	Not done	Ileo-colic fistula Crohn's disease
90	-	-	-	Not done	Surgical loop. Precise site not known

Results expressed as numbers of viable organisms per ml. aspirate

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

Table 11

FAECAL TYPE FLORA IN STOMACH REMNANT AND JEJUNUM OF TWENTY-SIX PATIENTS WITH PARTIAL GASTRECTOMY

No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Years since op.	Serum Vit. B_{12} µµg./ml.	Vitamin B_{12} Absorption	Diarrhoea	Stool fat Gms/day	Acid Secretion mEq.
91	Escherichia Cl. wechii Lactobacilli	Not done Not done Not done	1×10^5 1×10^6 8×10^3	16	62	Abnormal	No	10.4	0
92	Escherichia Lactobacilli	3×10^4 3×10^3	2×10^6 3×10^3	16	546	Normal	Yes	5.4	2.8
93*	Escherichia	-	9×10^5	7	589	Normal	No	Not done	0
94*	Proteus	2×10^3	2×10^3	22	Not done	Abnormal	No	6.2	0
95*	-	-	-	10	Not done	Abnormal	No	Not done	0
96*	Escherichia Citrobacter	1×10^7 5×10^2	6×10^6 8×10^5	10	Not done	Abnormal	No	4.0	0
97*	Escherichia	2×10^4	3×10^3	11	395	Abnormal x 2	No	3.4	1.3
98*	Escherichia Strep. faecalis	6×10^4 3×10^3	3×10^3 -	17	81	Abnormal	No	Not done	0
99	Klebsiella Lactobacilli	4×10^4 3×10^5	4×10^4 2×10^4	16	<50	Abnormal	No	3.1	0
100	Escherichia	2×10^5	1×10^6	8	<50	Abnormal	No	Not done	0
101	-	-	-	12	110	Normal	No	Not done	Not done
102	Lactobacilli	5×10^3	2×10^4	13	525	Normal	No	1.4	0
103	Escherichia Strep. faecalis Lactobacilli	8×10^6 7×10^5 6×10^5	3×10^5 2×10^5 2×10^5	14	287	Normal	No	6.2	0
104	Escherichia Klebsiella Lactobacilli	4×10^5 2×10^5 4×10^5	2×10^5 - 3×10^3	11	152	Abnormal	No	8.3	0
105	Proteus Lactobacilli	Not done Not done	9×10^3 6×10^3	10	985	Normal	No	6.5	0
106	Lactobacilli	3×10^5	1×10^3	15	190	Normal	No	2.5	1.6
107	-	-	-	9	452	Normal	No	5.7	4.1
108	-	-	-	9	350	Normal	No	5.0	2.8
109	-	-	-	9	281	Normal	No	10.9	0
110	-	-	-	9	201	Normal	No	2.3	0
111	-	-	-	9	365	Normal	No	6.7	0
112	Escherionia	2×10^5	5×10^5	8	Not done	Normal	No	6.4	0
113	Escherichia Lactobacilli	2×10^4 4×10^5	4×10^7 3×10^3	9	Not done	Abnormal	No	10.3	0
114	Escherichia Lactobacilli	5×10^6 2×10^6	5×10^5 1×10^3	8	98	Abnormal	No	2.1	0
115	Bacteroides Lactobacilli	2×10^5	4×10^4 1×10^4	8	129	Normal	No	5.2	0
116	Bacteroides -	-	5×10^5 -	8	546	Normal	No	4.6	0

Results expressed as numbers of viable organisms per ml. aspirate
- in columns 2, 3 and 4 indicate organisms present in concentration of less than 250/ml. aspirate

* Bacteroides and Lactobacilli not looked for

Table 12

FAECAL TYPE FLORA IN STOMACH REMNANT AND JEJUNUM OF TWENTY-ONE PATIENTS WITH GASTROENTEROSTOMY AND VAGOTOMY

No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Years since op.	Serum Vit. B_{12} µµg./ml.	Vitamin B_{12} Absorption	Diarrhoea	Stool fat Gms/day	Acid Secretion mEq.
117	Escherichia	8×10^7	8×10^7	2	138	Abnormal	Yes	5.1	0
118	Escherichia Lactobacilli	3×10^4 8×10^3	8×10^7 5×10^2	2	283	Abnormal	Yes	13.0	0
119	Citrobacter Lactobacilli	- 2×10^7	2×10^4 1×10^3	4	366	Normal	No	3.7	2.8
120	Lactobacilli	5×10^3	5×10^2	3	452	Normal	Intermittent	0.8	Not done
121	Citrobacter Lactobacilli	- -	2×10^3 2×10^3	3	429	Normal	No	6.3	34.2
122	Escherichia Strep. faecalis	3×10^3 -	7×10^7 3×10^9	5	246	Normal	No	15.0	8.8
123	Escherichia Klebsiella Lactobacilli	- - 8×10^2	5×10^6 5×10^6 -	1	375	Normal	Yes	3.4	0
124	Escherichia Strep. faecalis Lactobacilli	6×10^7 2×10^7 8×10^5	8×10^7 9×10^7 9×10^6	5	492	Abnormal	No	5.5	0
125	Lactobacilli	3×10^4	4×10^4	4	622	Normal	No	8.0	0
126	Lactobacilli	7×10^6	8×10^3	4	939	Normal	No	4.1	2.6
127	Lactobacilli	1×10^5	-	4	502	Normal	No	6.5	19.5
128	-	-	-	7	924	Normal	No	8.0	0
129	Lactobacilli	2×10^4	-	7	843	Normal	No	Not done	Not done
130	Escherichia Lactobacilli	- 8×10^3	5×10^5 -	7	513	Normal	No	Not done	4.9
131	Lactobacilli	5×10^4	-	6	457	Normal	No	4.9	2.5
132	Lactobacilli	4×10^5	5×10^2	2	1000	Normal	Yes	7.0	25.5
133	Escherichia	3×10^2	5×10^3	6	197	Normal	No	6.6	0
134	Escherichia Lactobacilli	2×10^5 2×10^5	1×10^8 1×10^8	5	131	Abnormal	No	3.2	0
135	Escherichia Alk. Dispar Lactobacilli	- - 7×10^5	2×10^6 1×10^5 7×10^5	5	333	Normal	No	17.6	1.0
136	-	-	-	6	678	Normal	Intermittent	8.2	8.4
137	-	-	-	5	373	Normal	No	3.4	10.9

Results expressed as numbers of viable organisms per ml. aspirate

- in columns 2, 3 and 4 indicate organisms present in concentration of less than 250/ml. aspirate.

gastric aspirates and one-half of the jejunal aspirates of all patients the counts were classified as abnormal, ranging from 2×10^4 to 3×10^9 viable organisms per ml. aspirate. Coliform organisms were particularly common, the most prominent group being Escherichia. Clostridium welchii were found only once in the jejunal juice of a patient with a partial gastrectomy. In only two patients were Bacteroides isolated. Lactobacilli were usually found in low concentrations only.

These were the largest groups of patients studied and the incidence of impaired vitamin B_{12} absorption and of steatorrhoea was high though the latter was only of a mild degree. Consequently an attempt was made to see if there was any relationship between these abnormalities and the finding of an abnormal bacteriology in terms of the findings of Enterobacteriaceae and Streptococcus faecalis. At the same time an attempt was made to assess the relation of gastric anacidity to the abnormal bacteriology.

In Table 13 the mean daily fat excretion in the stools has been related to the finding of a normal or abnormal faecal flora in the stomach, stomach remnant or jejunum of the patients. Presenting the results in this manner avoids the difficulty of having to define steatorrhoea and any bias which might result from this. It is seen that in both groups of patients fat excretion in the stools was higher when the flora was abnormal in the jejunum. The differences are, however, small and not statistically significant.

TABLE 13

Stool fat after gastric surgery related to gastrointestinal flora

	After Gastroenterostomy			After Partial Gastrectomy		
	Nos. studied	Mean stool fat per day (g.)	Range	Nos. studied	Mean stool fat per day (g.)	Range
Gastric flora						
Normal	15	6.9 ± 4.2	(0.8 - 17.6)	11	5.2 ± 2.5	(1.4 - 10.9)
Abnormal	4	6.2 ± 4.0	(3.2 - 13.0)	9	5.9 ± 2.5	(2.1 - 10.3)
	19			20		
Jejunal flora						
Normal	11	5.8 ± 2.2	(0.8 - 8.2)	11	4.9 ± 2.3	(1.4 - 10.9)
Abnormal	8	8.1 ± 5.4	(3.2 - 17.6)	10	6.3 ± 2.7	(2.1 - 10.4)
	19			21		

Abnormal flora = 10^4 viable organisms per ml. aspirate and over

In Tables 14 and 15 the bacteriological findings in the two groups of patients have been related to the presence of diarrhoea, achlorhydria and impaired absorption of vitamin B_{12}. Diarrhoea occurred in only one patient with a partial gastrectomy and in six patients after gastroenterostomy with vagotomy. Patients with intermittent diarrhoea have been included as cases of diarrhoea in these tables. There was no statistically significant relationship between the finding of diarrhoea and the occurrence of an abnormal flora in either the stomach or the jejunum.

When the flora is studied in relation to gastric acidity it is seen that in the presence of acid, abnormal bacteriological findings were unusual and the great majority of patients with an abnormal flora in the stomach or jejunum had achlorhydria after either operation. The findings in the stomach after gastroenterostomy are statistically significant.[1] The main reason for significance is, however, the fact that the patients with normal acid secretion tend to have a normal flora since of the patients with achlorhydria, about fifty per cent. also have a normal flora and the same is noted after partial gastrectomy.

As far as impaired absorption of vitamin B_{12} is concerned there appears to be significant correlation in the stomach after partial gastrectomy[2], and in the stomach[3] and jejunum[4] after gastroenterostomy between the finding of impaired absorption and the presence of

Calculations using Chi-squared with Yates' correction for small numbers (Mounsey, 1952):

1. $X^2 = 4.3$; $0.05 > p > 0.02$. 2. $X^2 = 6.44$; $0.02 > p > 0.01$.
3. $X^2 = 23$; $0.001 > p$. 4. $X^2 = 4$; $0.05 > p > 0.02$.

TABLE 14

Number of patients with complications after partial gastrectomy and their relation to gastrointestinal flora

	Nos. studied	Diarrhoea	Achlorhydria	Impaired absorption of vitamin B_{12}
Gastric flora				
Normal	14	0	10*	2
Abnormal	11	1	9	8
	25			
Jejunal flora				
Normal	14	0	9*	4
Abnormal	12	1	11	7
	26			

Abnormal flora = 10^4 viable organisms per ml. and over.

*Gastric acidity in one patient with normal flora not determined.

TABLE 15

Numbers of patients with complications after gastroenterostomy and their relation to gastrointestinal flora

	Nos. studied	Diarrhoea	Achlorhydria	Impaired absorption of vitamin B_{12}
Gastric flora				
Normal	17	4	4*	0
Abnormal	4	2	4	4
	21			
Jejunal flora				
Normal	12	3	3*	0
Abnormal	9	3	5	4
	21			

Abnormal flora = 10^4 viable organisms per ml. aspirate and over

*Gastric acidity in two patients with normal flora not determined

abnormal bacteriological findings. These findings must be interpreted with caution in view of the effects of acid which have been described above. Because of this the findings were reanalysed in cases with gastric achlorhydria and are illustrated in Table 16. The figures are no longer significant.

The above findings have been analysed without regard to the finding of lactobacilli. Taking these into consideration either on their own or together with the other abnormal findings, however, fails to produce any statistically significant relationships.

Patients with disorders of the liver

The results in these patients are illustrated in Table 17. In nine of these fifteen patients an abnormal faecal type flora was present in the jejunum. Patient No. 143 had portosystemic encephalopathy at the time of study and also had histamine-fast achlorhydria. Patients Nos 147 - 152 were studied in India and underwent more limited bacteriological investigations. These and patients Nos 143 and 144 were quite ill at the time of the study.

TABLE 16

Vitamin B_{12} absorption in achlorhydric patients after gastric surgery related to gastrointestinal flora

	After Gastroenterostomy		After Partial Gastrectomy	
	Nos. studied	Nos. with impaired absorption of Vit. B_{12}	Nos. studied	Nos. with impaired absorption of Vit. B_{12}
Gastric Flora				
Normal	4	0	9	2
Abnormal	3	3	9	7
	7		18	
Jejunal Flora				
Normal	3	0	9	3
Abnormal	5	4	11	7
	8		20	

Abnormal flora = 10^4 viable organisms per ml. aspirate and over

TABLE 17

Faecal type flora in stomach and jejunum of fifteen patients with disorders of the liver

Case No.	Organisms found	Gastric Aspirate	Jejunal Aspirate	Serum Albumin Gm/100 ml.	Ascites	Oesophageal Varices	Diagnosis
138	Lactobacilli	6×10^5	2×10^3	3.2	No	No	Obstructive Jaundice
139	Strep. faecalis	-	5×10^4	2.5	No	No	Recovering from Acute Virus Hepatitis
140	-	-	-	2.6	No	No	Chronic relapsing Hepatitis
141	Escherichia Strep. faecalis	Not done Not done	4×10^6 3×10^5	3.5	No	Yes	Cirrhosis
142	-	-	-	3.1	No	Yes	Cirrhosis (Alcoholic)
143	Escherichia Alkalescens-Dispar Citrobacter Strep. faecalis	- - - -	1×10^5 1×10^5 5×10^4 5×10^5	2.8	No	 Yes	Cirrhosis
144	Strep. faecalis Lactobacilli	Not done	2×10^4 4×10^4	1.8	Yes	Not done	Cirrhosis (Alcoholic) On Neomycin at time of study
145	Lactobacilli	8×10^4	-	2.8	No	No	Cirrhosis (Alcoholic) On Neomycin till two weeks before study
146	-	-	-	2.8	No	No	Cirrhosis
* 147	Escherichia	-	2×10^5	2.3	Yes	Not done	Cirrhosis (Nutritional)
* 148	Escherichia	2×10^2	5×10^4	2.5	Yes	Not done	" "
* 149	Escherichia	-	3×10^5	2.7	Yes	Yes	" "
* 150	-	-	-	2.6	Trace	Not done	" "
* 151	Escherichia	Not done	1×10^6	2.3	Yes	Not done	" "
* 152	Escherichia Strep. faecalis	- -	1×10^4 8×10^3	2.3	Yes	Not done	" "

Results expressed as numbers of viable organisms per ml. aspirate

- in columns 2, 3 and 4 indicates organisms present in concentration of less than 250 per ml. aspirate

* Lactobacilli and Bacteroides not looked for

The studies in vitro

(i) The study of general factors and of human gastric juice on vitamin B_{12} uptake by Escherichia.

Studies of the uptake of labelled cyanocobalamin were carried out with forty-four strains of organisms representing micro-organisms of the faecal type which were looked for in the gastro-intestinal tract. The results of these studies are indicated in Table 18. Enterobacteriaceae appear to be very avid for vitamin B_{12} though the Proteus group is somewhat less so. Strains of Streptococcus faecalis took up very little of the labelled vitamin. All the organisms on which these tests were performed had been isolated from the gastrointestinal tract except for the Cl. welchii (Fig. 2) for which studies control strains were obtained by courtesy of the Edinburgh University Department of Bacteriology.

It is seen from Figs 3, 4 and 5 that when the rates of uptake were measured using a 1 ml. inoculum of an overnight culture of the organism, on an average maximum uptake occurred after about five hours. The pattern of uptake by organisms obtained from patients with blind or stagnant loops was similar to that of organisms isolated from other patients. The number of Proteus strains isolated were few and six organisms obtained from the National Collection of Type Cultures (Colindale Avenue, London, N.W. 9) were therefore examined (Fig. 6). The results confirm that these organisms are less active

TABLE 18

Uptake of labelled cyanocobalamin by organisms isolated from the G.I. tract

	Organism genus	No. of strains	Mean % uptake of radioactivity	Range
1.	Escherichia	13	88.5%	78 - 98%
2.	Klebsiella	5	87%	80 - 93%
3.	Alkalescens-Dispar	3	87%	80 - 91%
4.	Citrobacter	1	84%	
5.	Cloaca	1	84%	
6.	Proteus*	3	70%	56 - 83%
7.	Streptococcus faecalis	9	6%	0 - 20%
8.	Cl. welchii	7	72%	50 - 84%
9.	Lactobacilli	2	80%	78 - 82%

*incl. Providencia

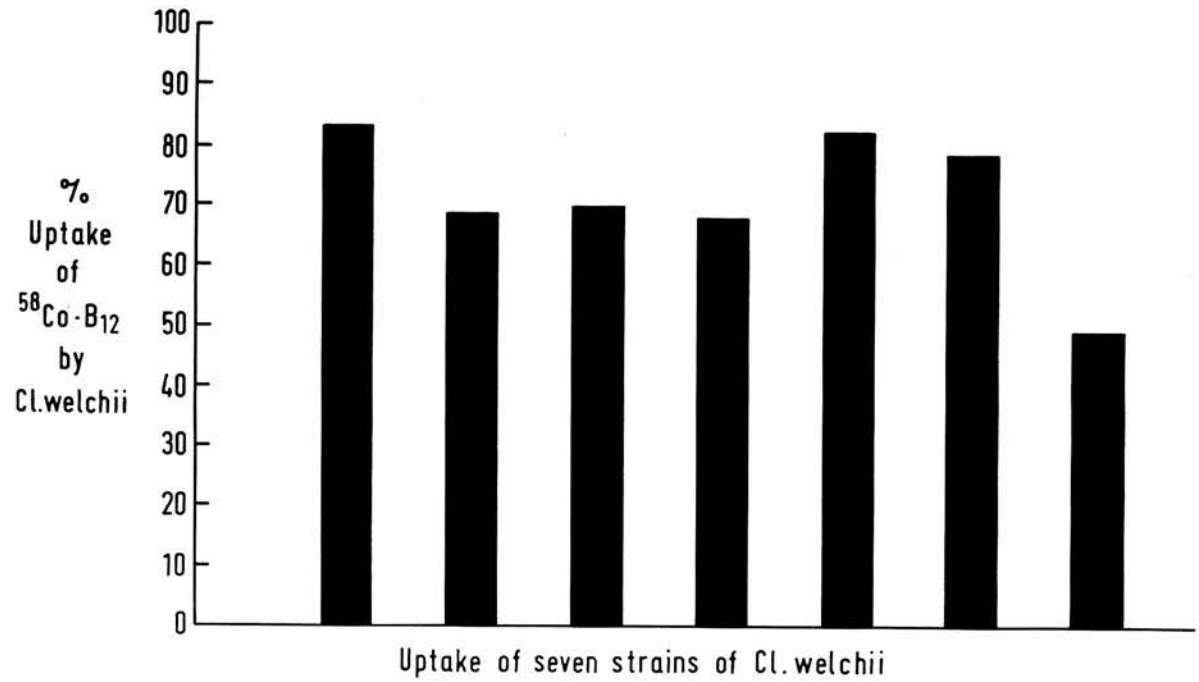


Figure 2. Uptake of vitamin B_{12} by seven strains of Clostridium welchii.

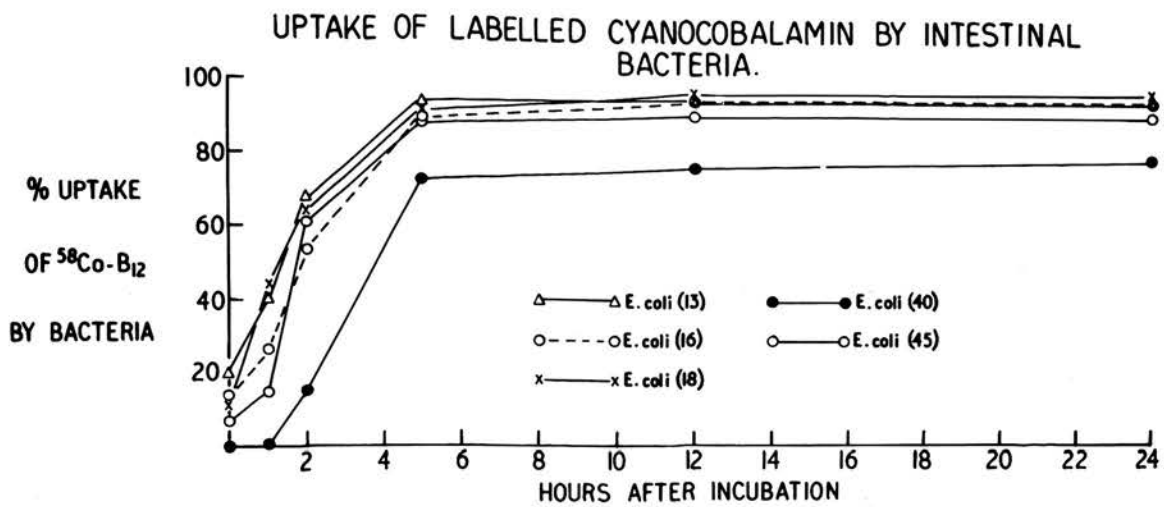


Figure 3. Uptake of vitamin B_{12} by intestinal bacteria. Numbers in brackets are author's reference to strain of organism.

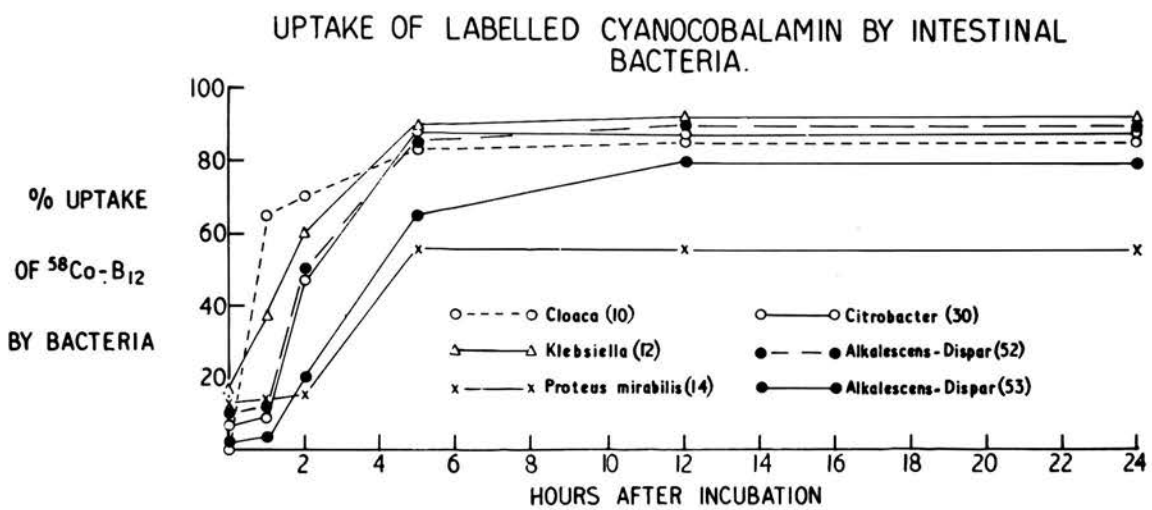


Figure 4. **Uptake of vitamin B_{12} by intestinal bacteria. Numbers in brackets are author's reference to strain of organism.**

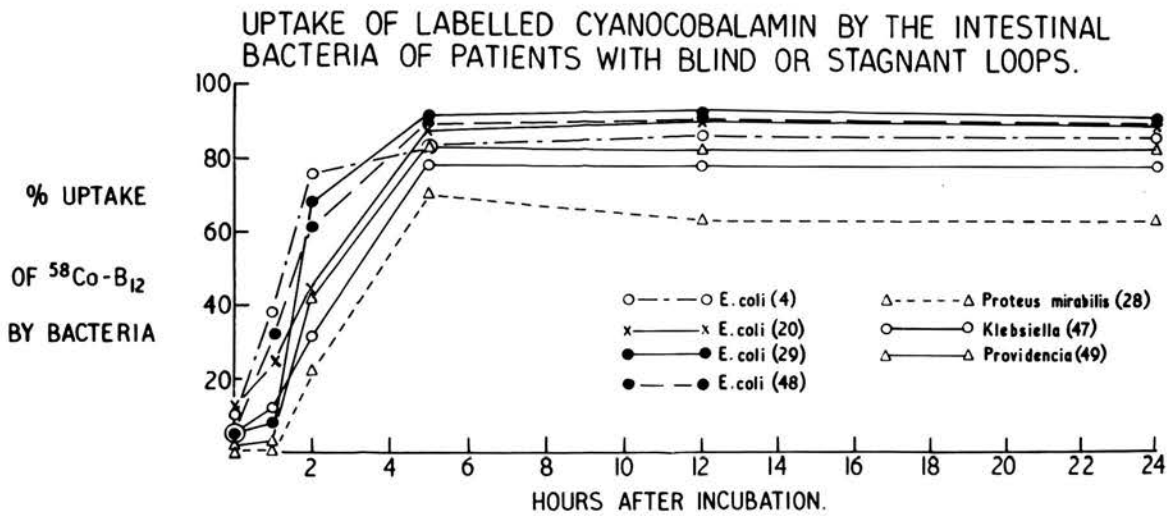


Figure 5. Uptake of vitamin B_{12} by intestinal bacteria of patients with blind or stagnant loops. Numbers in brackets are author's reference to strain of organism.

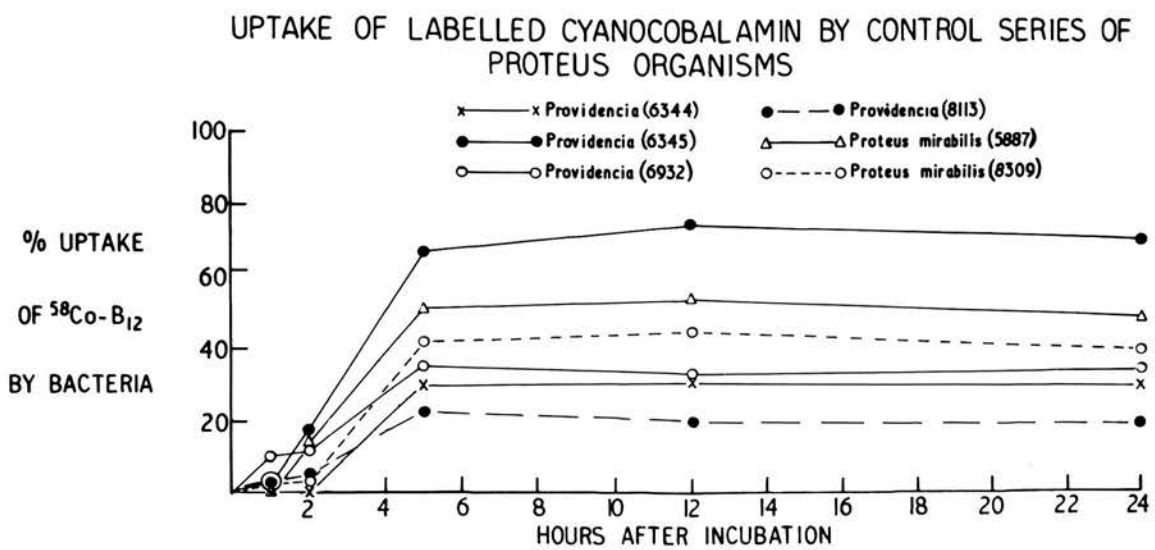


Figure 6. Uptake of vitamin B_{12} by control series of proteus organisms. Numbers in brackets refer to the catalogue number of the strain (Catalogue of Species, M.R.C. Memorandum No. 35)

in their uptake of cyanocobalamin.

In the *in vivo* situation it is much more likely that when organisms encounter vitamin B_{12} in the gastrointestinal tract they will do so in large numbers and will be actively growing. The effects of a four hour growing culture is illustrated in Fig. 7 where it is seen that the amount of vitamin B_{12} taken up immediately is very considerable and maximum uptake occurred much earlier.

In Fig. 8 is illustrated the effect of human gastric juice on the ability of *Escherichia coli* to remove vitamin B_{12} from the culture medium. In the absence of gastric juice a four hour growing culture took up about 80 - 90% of the vitamin in the medium after further incubation for one hour. As might be expected from Fig. 7 prolonging the period of incubation did not significantly increase this figure which recurs in subsequent experiments. It will be seen that even one ml. of gastric juice had a marked inhibitory effect on the ability of the coliform to take up the vitamin though this inhibition was not complete. As is seen from Fig. 9 the tonicity of the broth did not significantly affect the ability of the organisms to take up vitamin B_{12} either alone or in the presence of gastric juice.

The binding of vitamin B_{12} by gastric juice is known to be very rapid. This is illustrated in Table 19 in which juice of low intrinsic factor activity was employed. In Fig. 10 a comparison is made of the effects of adding vitamin B_{12} already exposed to gastric juice to a growing culture containing the gastric juice. Incubation was subsequently continued for one hour. The binding of the gastric

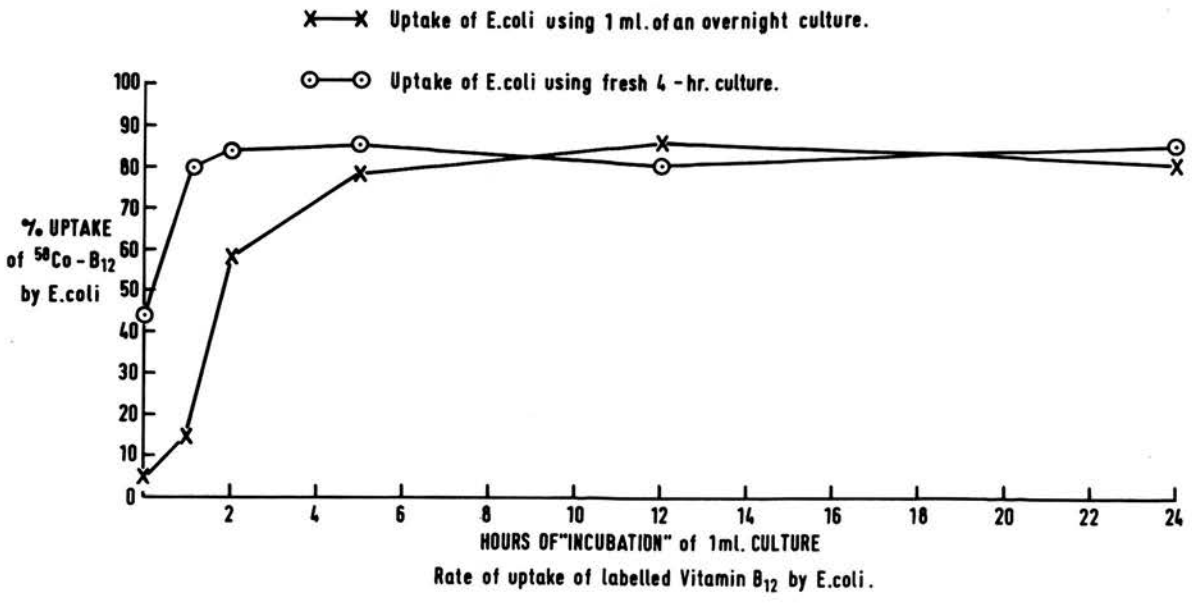


Figure 7. Rate of uptake of a 4 hr culture of E. coli compared to rate of uptake of a 1 ml. overnight culture.

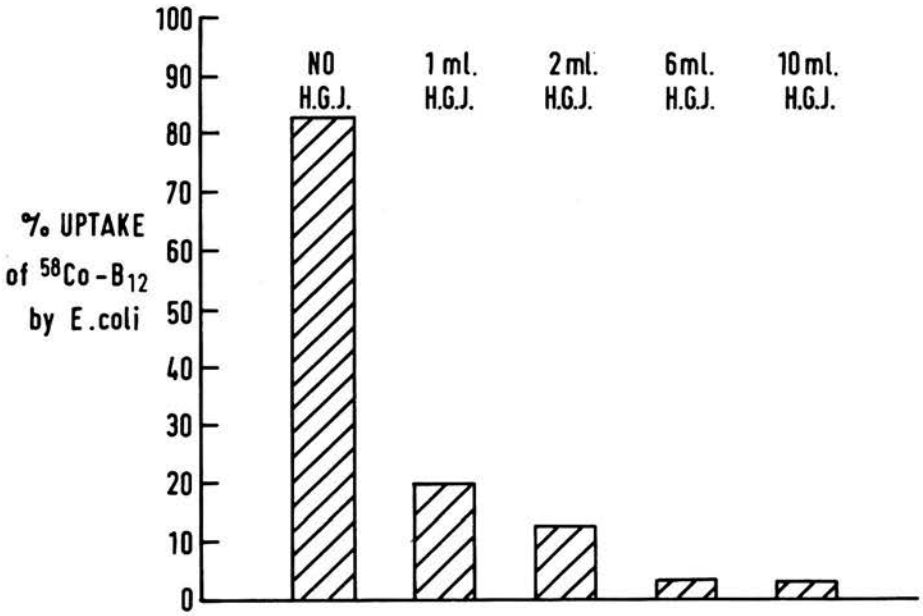


Figure 8. Uptake of growing culture of <u>E. coli</u> after 1 hr in the presence of various quantities of human gastric juice.

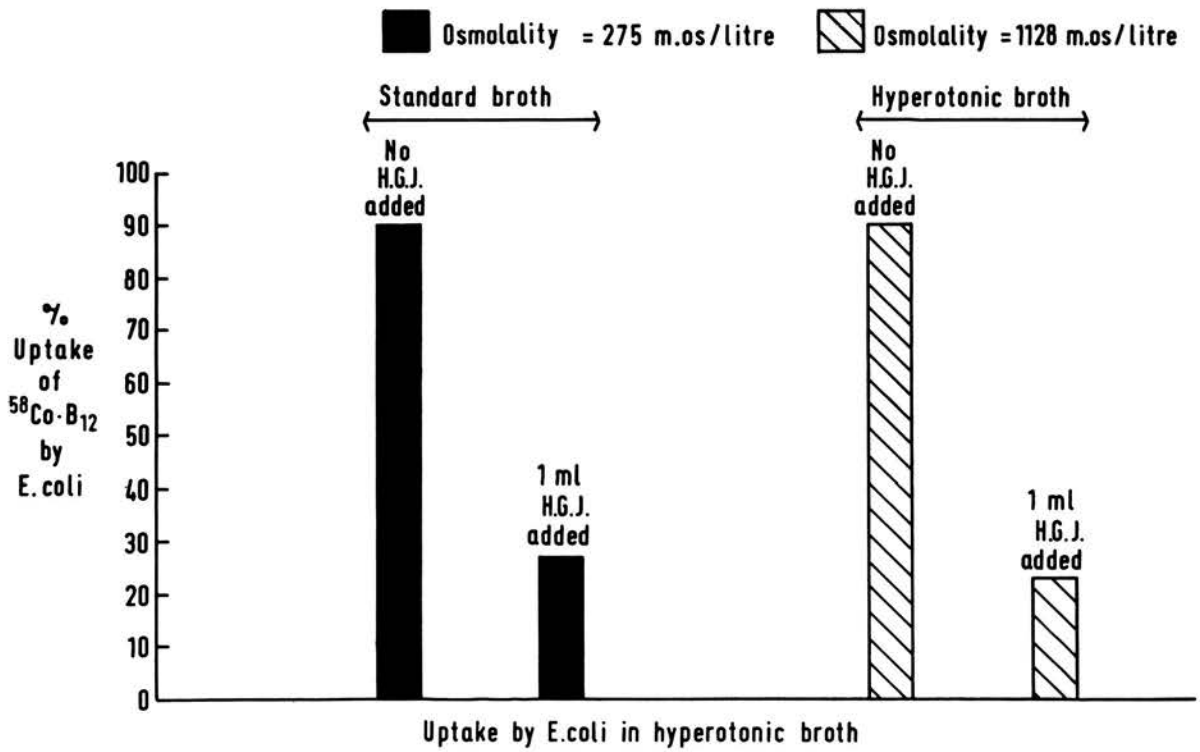


Figure 9. Uptake by E. coli in hypertonic broth.

TABLE 19

			% Uptake in 1 hr
1 ml. H.G.J. incubated to 37° C	- 1/15 ug. ^{58}Co-vit. B_{12} added	Add 4 hr growing culture <u>immediately</u>	- 73%
1 ml. H.G.J. incubated to 37° C	- 1/15 ug. ^{58}Co-vit. B_{12} added	Add 4 hr growing culture <u>after ½ hr.</u>	- 72%
1 ml. WATER incubated to 37° C	- 1/15 ug. ^{58}Co-vit. B_{12} added	Add 4 hr growing culture <u>immediately</u>	- 90%

Intrinsic factor concentration of juice 6.5 ngm. per ml.

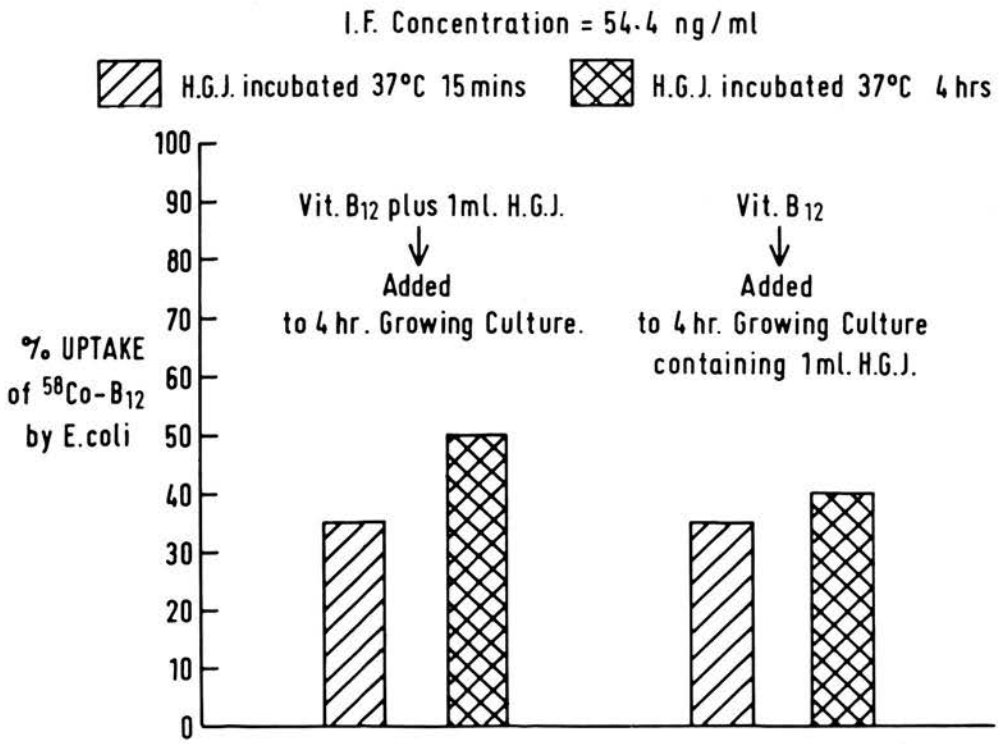


Figure 10. To determine if a growing culture of E. coli can compete with gastric juice for vitamin B_{12}.

juice present in the growing culture was just as marked as that of the gastric juice which came into contact with vitamin B_{12} before the vitamin came into contact with the culture. The slightly higher uptakes illustrated in the crosshatched columns show that gastric juice loses some binding activity with incubation for four hours even after its content of pepsin has been inactivated. This is also noted in subsequent experiments. In spite of this, however, gastric juice cannot bind vitamin B_{12} to which the microorganism has obtained first access. This is illustrated by Fig. 11 which compares the uptake by various 1 ml. overnight cultures and various 4 hour growing cultures to each of which gastric juice was added at different time intervals. Comparing the uptakes in these experiments with the curves in Fig. 7 suggests that the gastric juice merely prevents further uptake by the coliform but cannot bind vitamin taken up to the point of addition. The results illustrated in Fig. 12 in which parallel samples were withdrawn when gastric juice was added at two and six hours confirm this and also that large volumes of gastric juice are no more able to bind vitamin B_{12} to which organisms have obtained first access than are the smaller volumes.

Two factors likely to be operating in the uptake of vitamin B_{12} are growth and numbers of organisms. From Fig. 13 it will be seen that a 24 hr 10 ml. culture exhibited poor uptake compared to that of a four hour culture even when incubation was prolonged. The young four hour cultures could also withstand considerable dilution with no loss of ability to take up the vitamin. Heating to 56° C (Fig. 14)

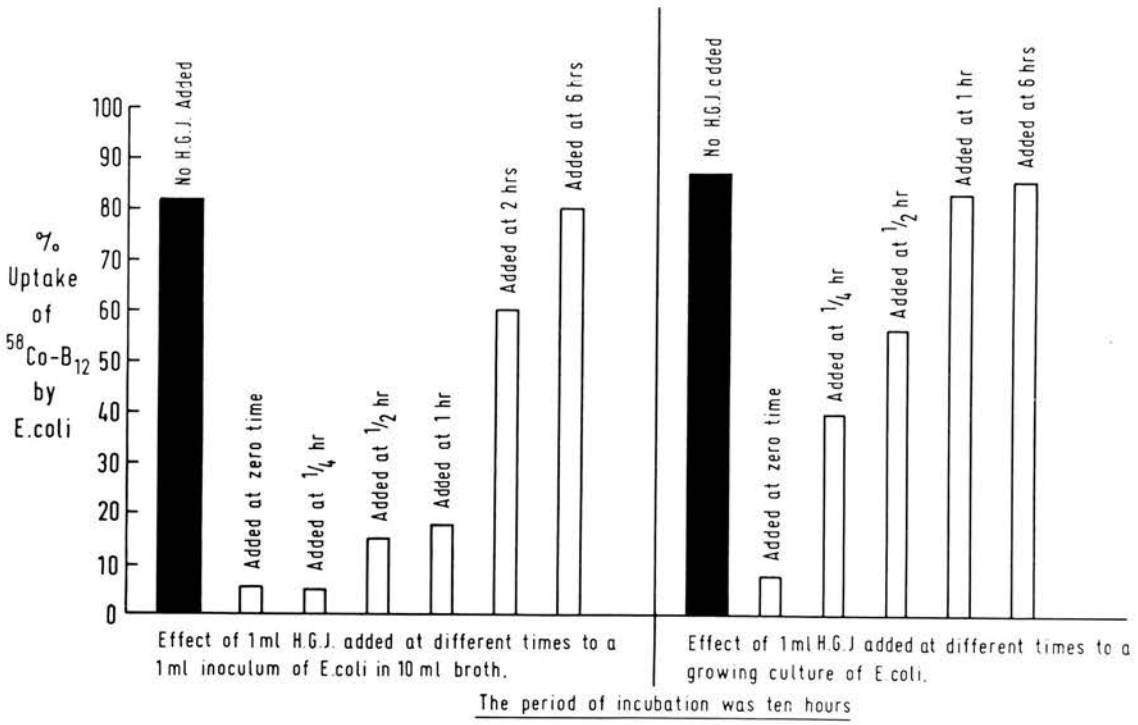
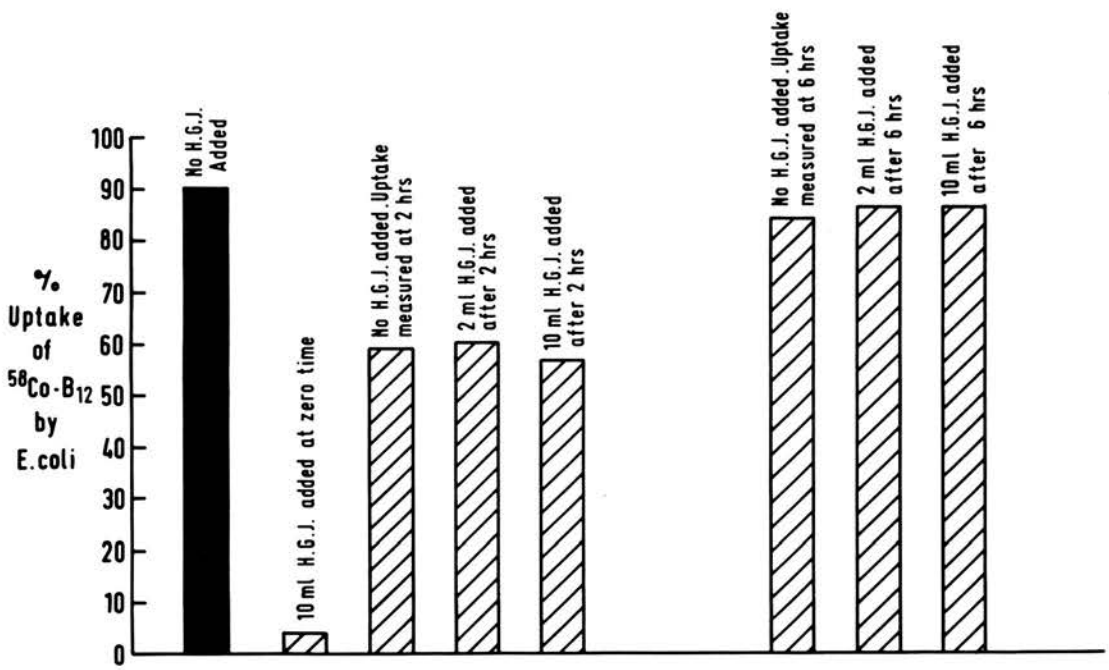


Figure 11. **Effect of 1 ml. of human gastric juice added at different times to a 1 ml. inoculum of E. coli and a growing culture of E. coli.**



Effect of adding H.G.J. at 2 and 6 hrs to a 1 ml inoculum of E.coli in 10 ml broth.
Period of incubation was ten hours except in cultures withdrawn at 2 and 6 hours

Figure 12. Effect of adding human gastric juice at 2 and 6 hours to a 1 ml. inoculum of E. coli in 10 ml. broth.

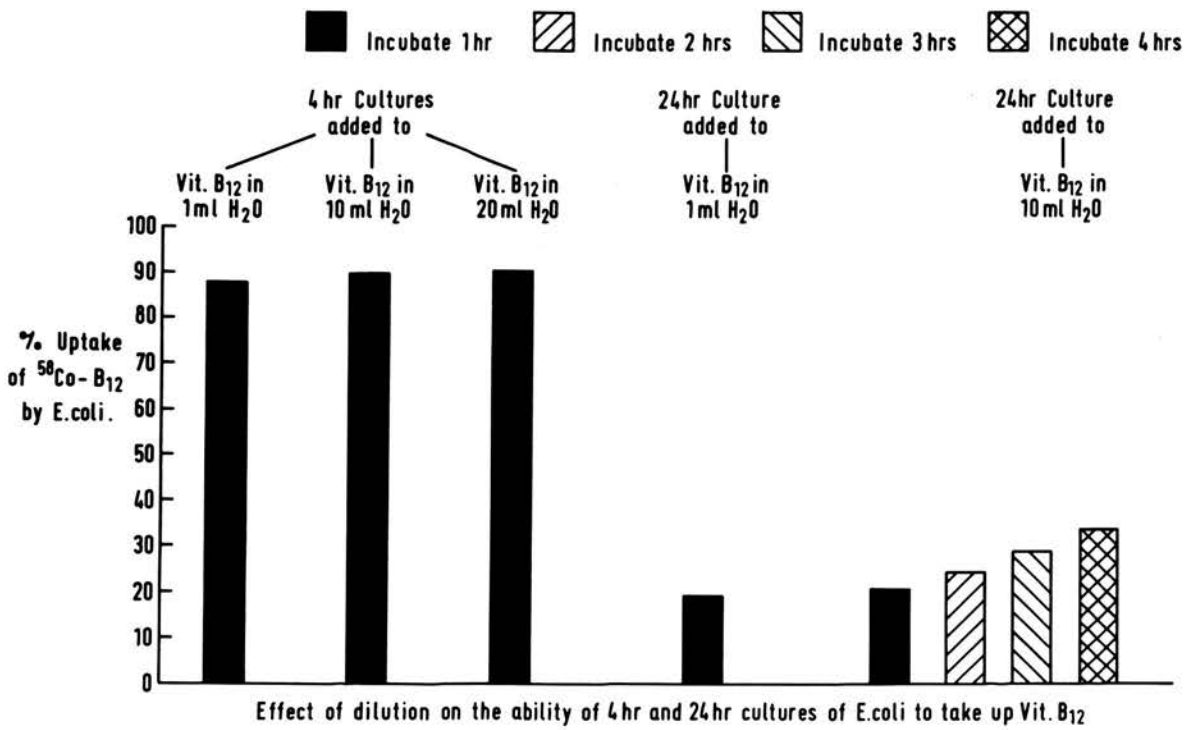


Figure 13. Effect of dilution on the ability of 4 hr and 24 hr cultures of E. coli to take up vitamin B_{12}.

had a marked inhibitory effect on the uptake of the four hour cultures but none on the 24 hr cultures. The heated cultures were shown not to be dead.

In Fig. 15 are illustrated the results of a study in which five and 20 ml. cultures grown for four, seven and 24 hours were added to labelled cyanocobalamin in one ml. of water or one ml. of gastric juice of low intrinsic factor concentration. After incubation for a further hour uptake was maximal and similar in all the four hour cultures. Since the five and the 20 ml. cultures were inoculated simultaneously their content of organisms, which would be in the logarithmic phase of growth, was likely to be similar. The 24 hr cultures would contain more organisms than the younger cultures but their ability to take up vitamin B_{12} was poor though about four times greater in the 20 ml. than in the five ml. cultures. In the seven hour cultures vitamin B_{12} uptake was intermediate between that of the four and 24 hr cultures.

It is tempting to suggest that the greater uptake by the younger cultures reflects the fact that these organisms are in the logarithmic as distinct from the stationary phase of growth of the older cultures. Further work is planned to try and elucidate this. The pH of the older cultures falls to 5 and less and if this is raised the uptake of vitamin B_{12} by these cultures rises to that of the four hour cultures within two hours. Low pH may be operating by inhibiting significant multiplication during the stationary phase or by some other more specific method. On the other hand it is possible that raising the

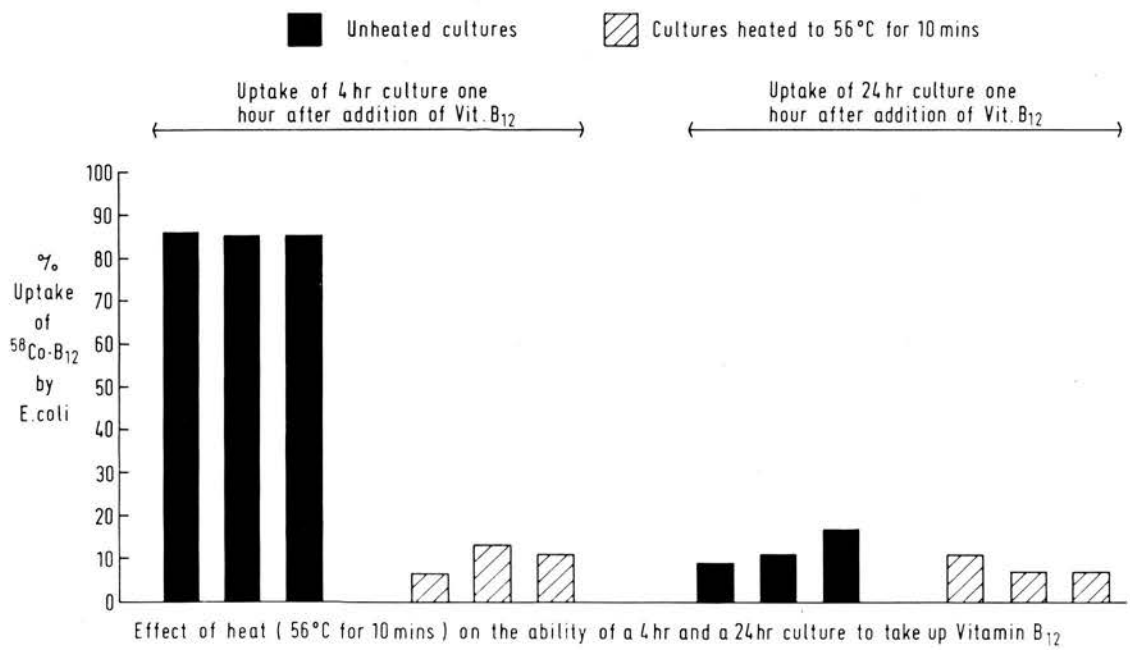
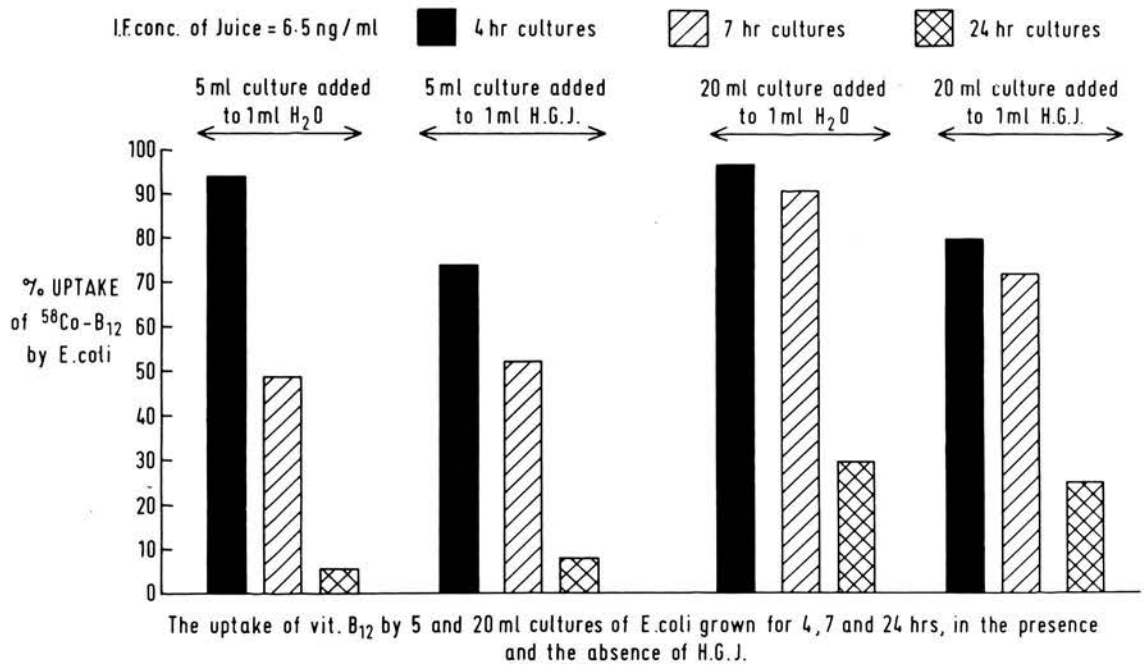


Figure 14. Effect of heat (56° C for 10 min.) on the ability of a 4 hr and a 24 hr culture of E. coli to take up vitamin B_{12}.



The uptake of vit. B_{12} by 5 and 20 ml cultures of E.coli grown for 4, 7 and 24 hrs, in the presence and the absence of H.G.J.

Figure 15. Uptake of vitamin B_{12} by 5 and 20 ml. cultures of E. coli grown for 4, 7 and 24 hours in the presence and absence of human gastric juice.

pH allows organisms to go into a new logarithmic phase. It is also likely that the effects of heat shock noted in Fig. 14 are due to inhibition of growth. Though some organisms would die as a consequence of this treatment it is unlikely that vitamin B_{12} released into the medium as a consequence of their death affects the results in the light of work illustrated in Fig. 16. Thus three times the amount of vitamin employed in these studies was unable to saturate the ability of growing four hour cultures to take up vitamin B_{12}. The greater uptake with increasing amounts of vitamin in the presence of gastric juice is due to saturation of the binding ability of the gastric juice.

An attempt to illustrate the combined effects of growth and increased numbers of organisms on vitamin previously exposed to gastric juice is illustrated in Figs 17 and 18 which indicate the results of two experiments in which the uptake by Escherichia of the vitamin was again determined in the presence of one ml. of gastric juice. When uptake was measured after adding the 10 ml. four hour cultures to the juice no significant increase in uptake occurred with incubation to five and 10 hours over the uptake obtained at one hour. This is what would be expected from the observations illustrated in Fig. 7 in the absence of gastric juice. When, however, extra nutrients at pH 6.8 were added at one and five hours the organisms took up more of the vitamin. The extra nutrients were in the form of 6 ml. of medium which was free from vitamin B_{12} (medium used for the microbiological assay of vitamin B_{12} using Lactobacillus leichmannii as the

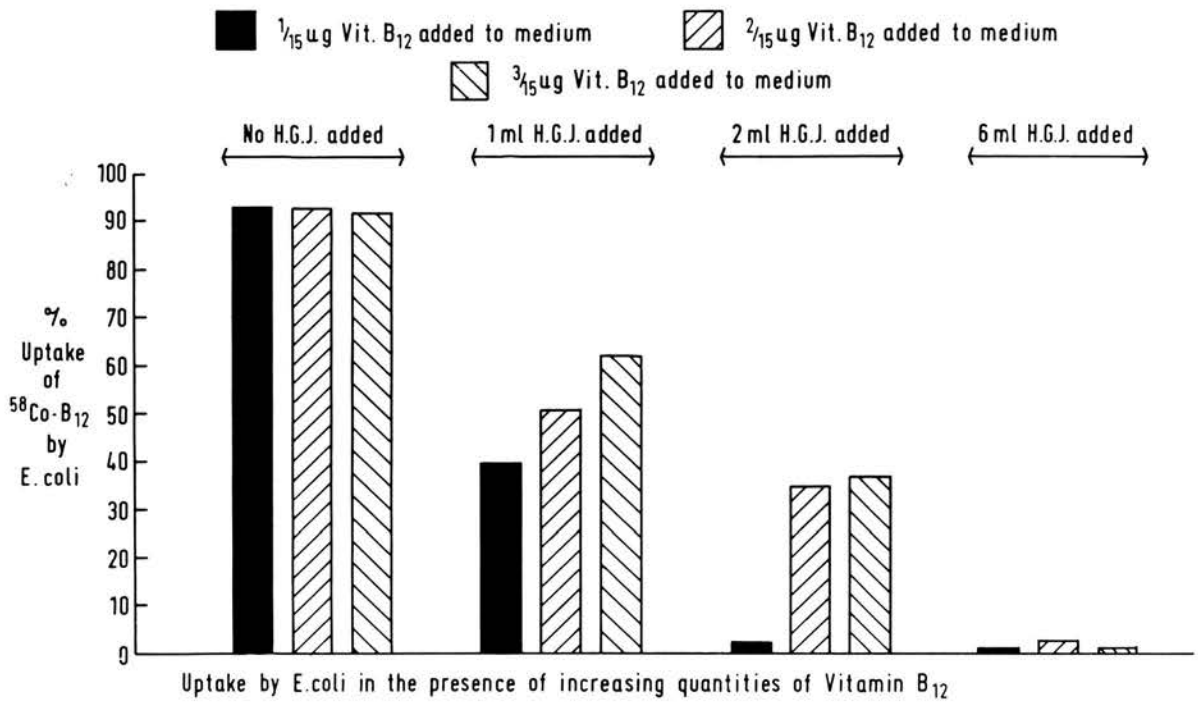
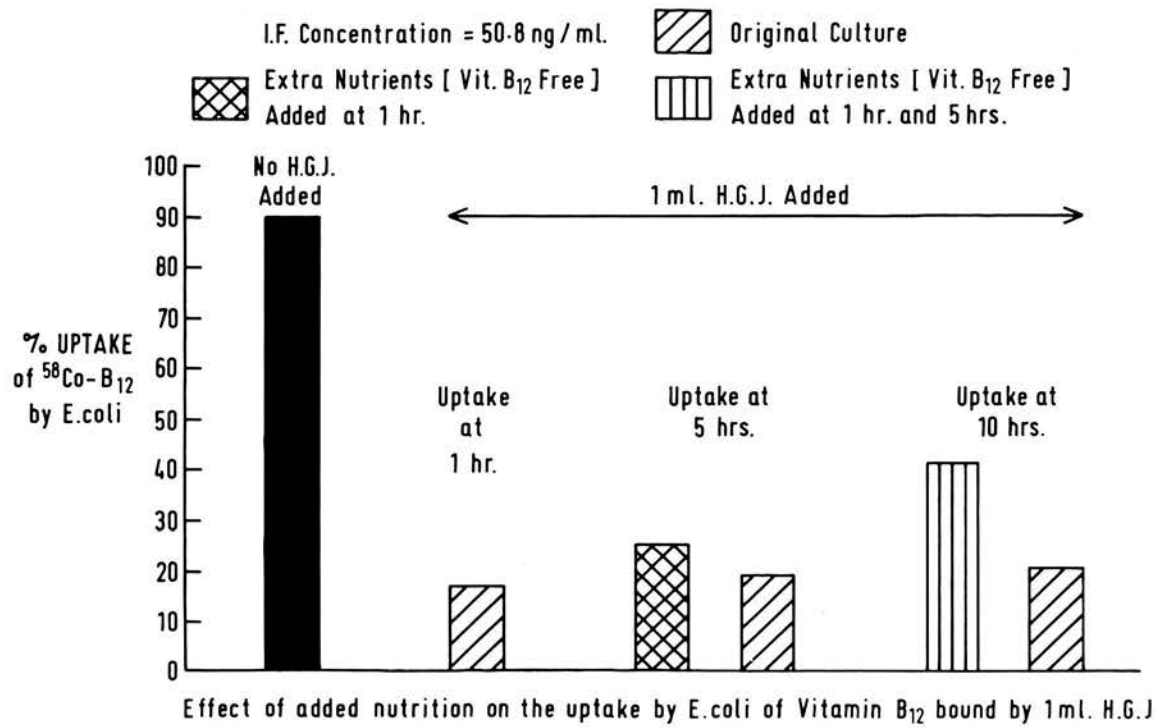


Figure 16. Uptake by *E. coli* in the presence of increasing quantities of vitamin B_{12}.



Effect of added nutrition on the uptake by E.coli of Vitamin B_{12} bound by 1 ml. H.G.J.

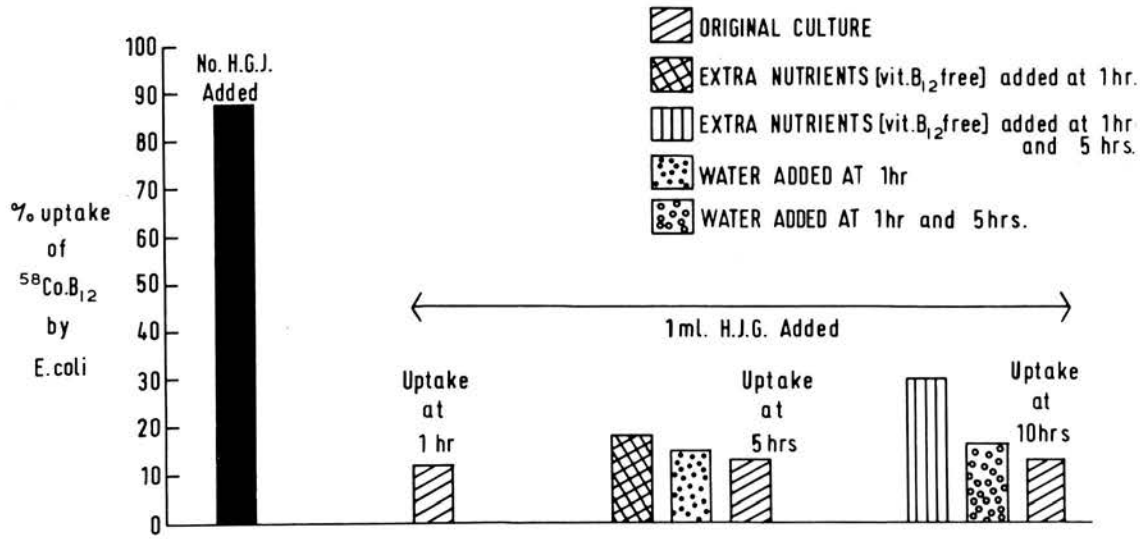
Figure 17. **Effect of added nutrition on the ability of <u>E. coli</u> to take up vitamin B_{12} in the presence of 1 ml. of human gastric juice.**

test organism). Vitamin B_{12} free medium was used to prevent error which might have arisen from exchange between bound and unbound vitamin B_{12} (Donaldson and Katz, 1962). That the results obtained are not merely due to dilution is seen from the poor uptake exhibited by the cultures to which six ml. of water were added instead of nutrients (Fig. 18). Optical density measurements made on parallel cultures in this experiment confirmed that growth occurred in the presence of the added nutrients (Table 20).

In the context of previous arguments in relation to pH it will be seen from Fig. 19 that Escherichia are capable of taking up vitamin B_{12} in a pH range of 5 - 9. Here the pH of the four hour 10 ml. cultures was altered as indicated and added to the vitamin B_{12} contained in one ml. of water or one ml. of gastric juice at identical pH. It is somewhat surprising that a large volume of organisms is unable to take up any significant vitamin at low pH. It is possible that quite apart from an inhibitory effect on the growth of the organisms, the manner in which vitamin B_{12} is assimilated by the organism is specifically blocked at the low pH levels.

(ii) The effect of crystalline enzymes on the binding activity of human gastric juice.

The effect of crystalline pepsin on the binding activity of the gastric juice is illustrated in Fig. 20. Four hour growing cultures were added to enzyme digested and to undigested juice,



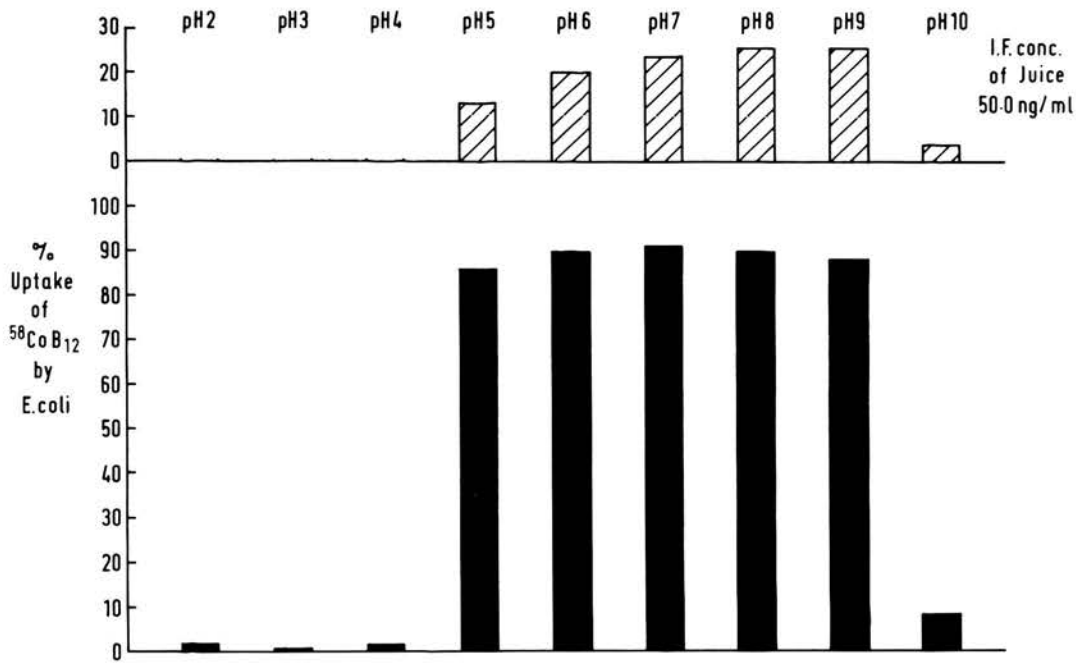
Effect of dilution and added nutrition on the ability of E.coli to take up vitamin B_{12} in the presence of 1ml of H. G. J.

Figure 18. Effect of dilution and added nutrition on the ability of <u>E. coli</u> to take up vitamin B_{12} in the presence of 1 ml. of human gastric juice.

TABLE 20

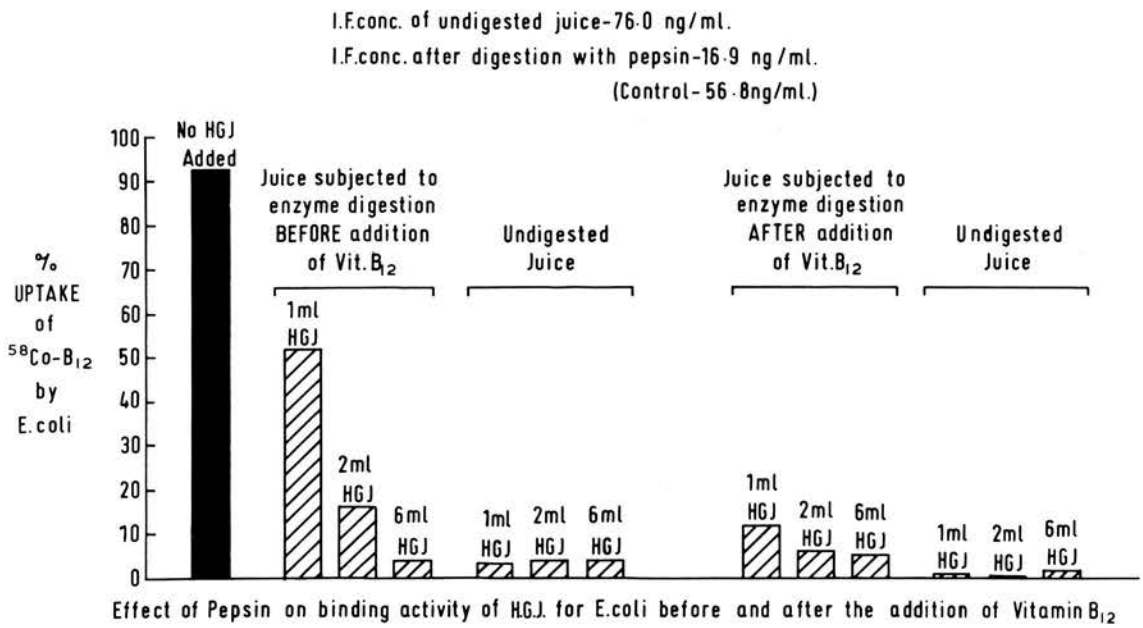
O.D. of original culture at 1 hr.	140
O.D. of original culture plus 12 ml. L. leichmannii medium added at 1 hr.	78
O.D. of original culture plus 12 ml. water added at 1 hr. ..	74
O.D. of original culture at 10 hr. - 6 ml. L. leichmannii medium added at 1 hr. and at 5 hr.	94
O.D. of original culture at 10 hr. - 6 ml. water added at 1 hr. and at 5 hr.	76

Optical density measurements for Fig. 18.



Effect of adding growing culture of E.coli at various pH values to Vit. B_{12} in 1 ml H.G.J. (above) and 1ml H_2O (below)

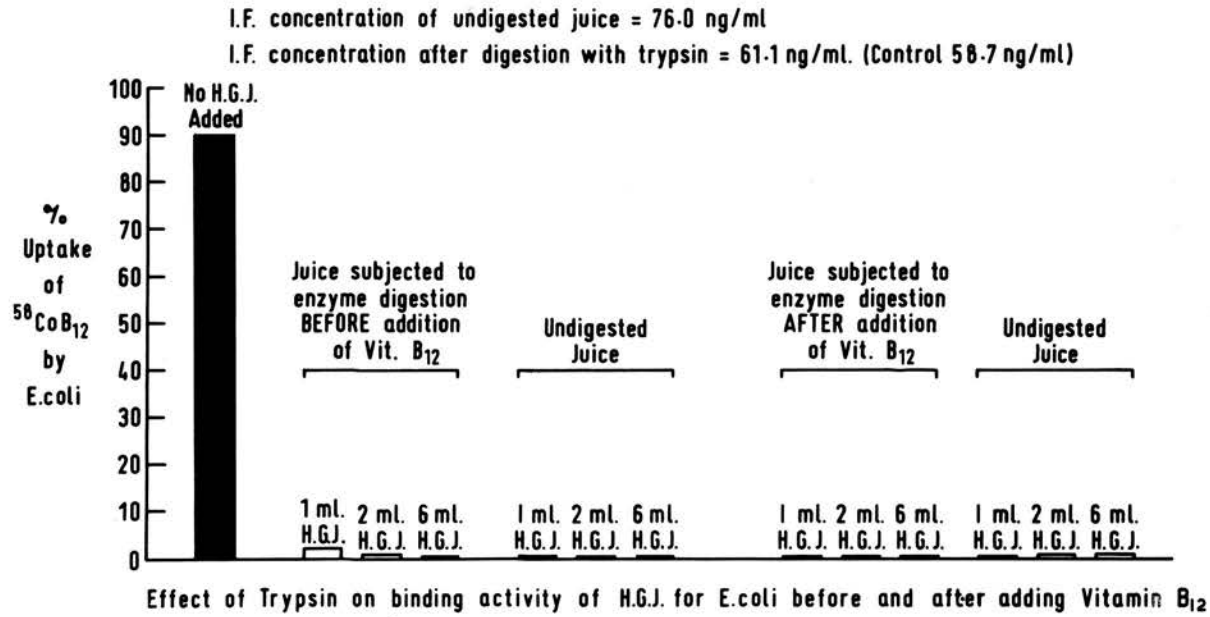
Figure 19. Effect of adding growing cultures of E. coli at various pH values to vitamin B_{12} in 1 ml. of human gastric juice and 1 ml. of water.



Effect of Pepsin on binding activity of H.G.J. for E.coli before and after the addition of Vitamin B_{12}

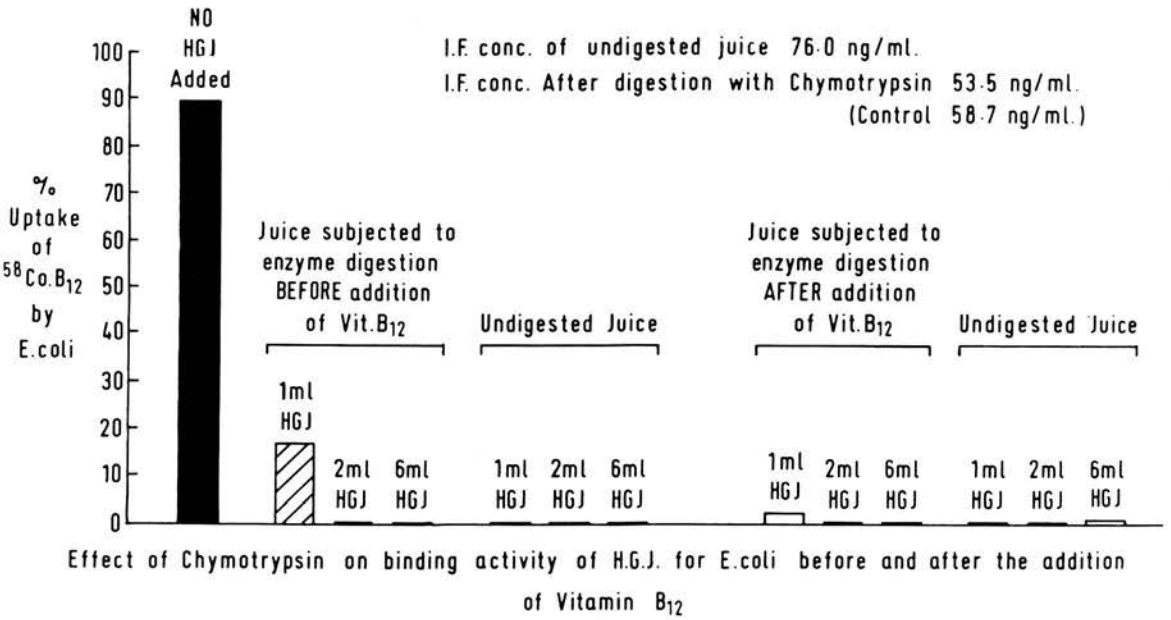
Figure 20. Effect of Pepsin on binding activity of human gastric juice for E. coli before and after the addition of vitamin B_{12}.

incubation at 37° C being continued for a further hour. One ml. of gastric juice showed marked loss of binding activity after peptic digestion and it appears from the intrinsic factor concentrations of the juice before and after digestion that the main reason for the loss of binding activity is the fall in the intrinsic factor concentration of the gastric juice. The digestive effect of pepsin on the binding activity of the gastric juice was much less significant when the vitamin was added to the juice prior to the addition of the enzyme and this is in keeping with what is known of the greater resistance of intrinsic factor to enzyme digestion after prior exposure to vitamin B_{12} (Gräsbeck, 1959a). The method employed to assay intrinsic factor does not permit determinations in those experiments in which vitamin B_{12} was added to the gastric juice prior to enzyme digestion. The results using trypsin and chymotrypsin alone and in various combinations which include pepsin are illustrated in Figs 21 - 26. There is some suggestion that chymotrypsin had some effect on binding activity (Fig. 22). On the whole, however, these enzymes appear to add little to the effect of pepsin. Though there was some slight suggestion of loss of binding activity when they were used in combination with pepsin, examination of the intrinsic factor estimations show no significant fall over the values in the juice used as controls to which no enzyme was added, and the effects may therefore be the consequence of digestion of vitamin B_{12} binders other than intrinsic factor. As might be expected, the effect of crystalline enzymes on the binding activity of the juice was more



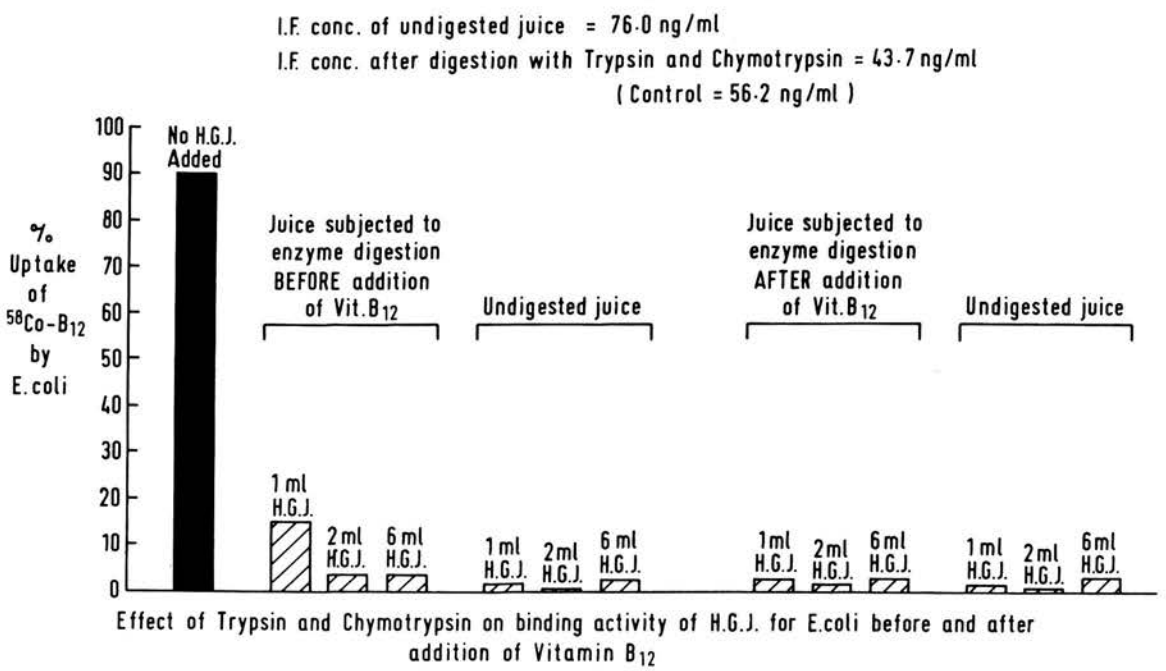
Effect of Trypsin on binding activity of H.G.J. for E.coli before and after adding Vitamin B_{12}

Figure 21. Effect of Trypsin on binding activity of human gastric juice for E. coli before and after adding vitamin B_{12}.



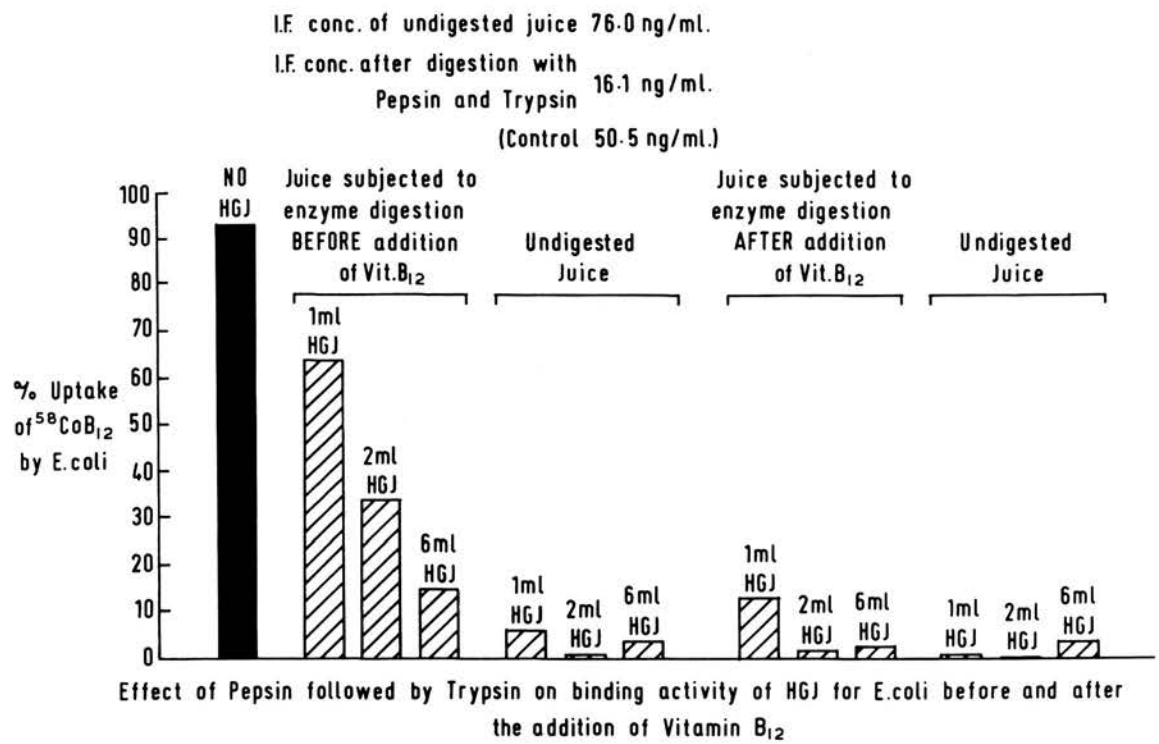
Effect of Chymotrypsin on binding activity of H.G.J. for E.coli before and after the addition of Vitamin B_{12}

Figure 22. Effect of Chymotrypsin on binding activity of human gastric juice for E. coli before and after adding vitamin B_{12}.



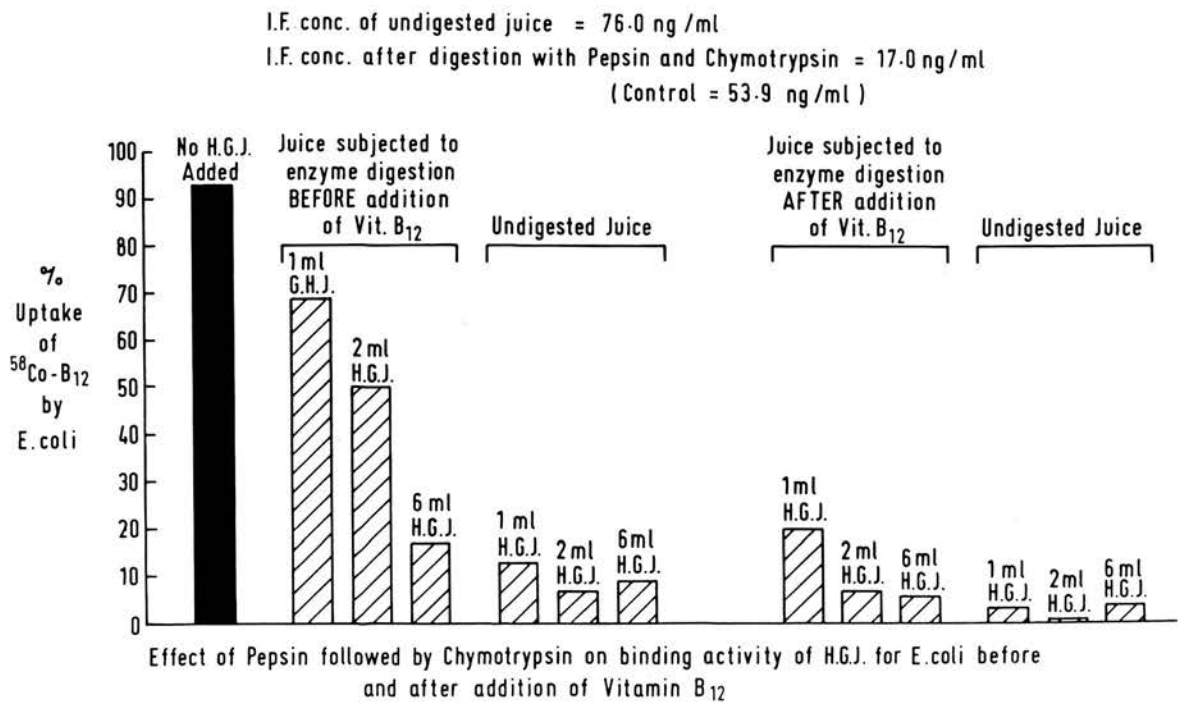
Effect of Trypsin and Chymotrypsin on binding activity of H.G.J. for E.coli before and after addition of Vitamin B_{12}

Figure 23. Effect of Trypsin and Chymotrypsin on binding activity of human gastric juice for E. coli before and after adding vitamin B_{12}.



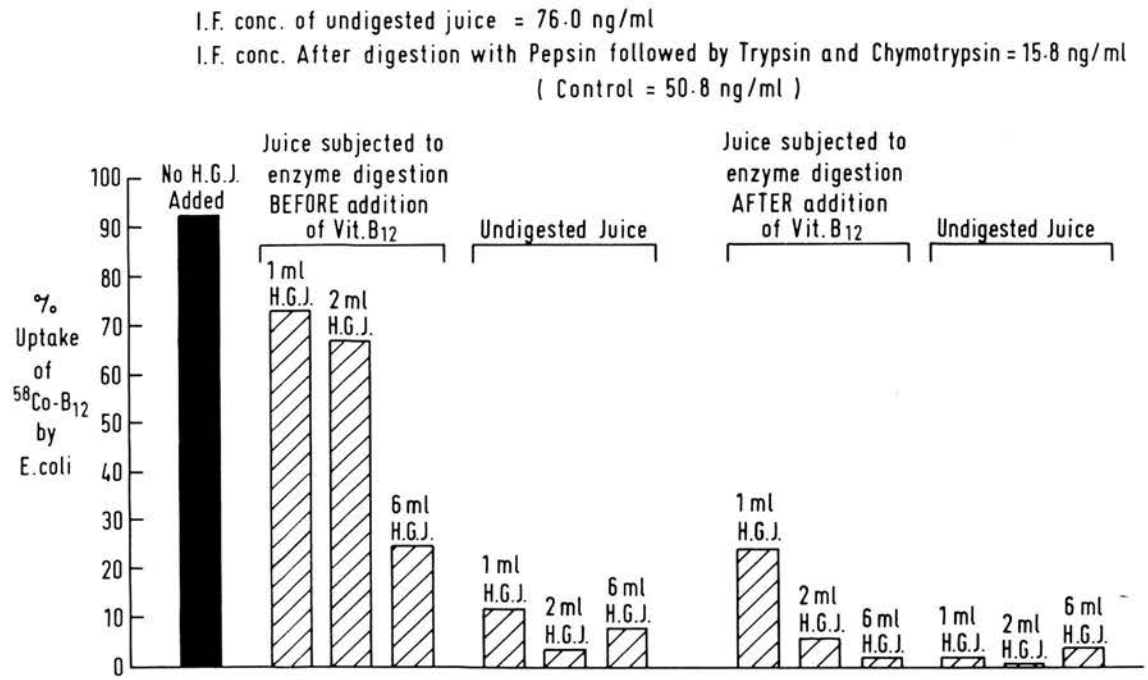
Effect of Pepsin followed by Trypsin on binding activity of HGJ for E.coli before and after the addition of Vitamin B_{12}

Figure 24. Effect of Pepsin followed by Trypsin on binding activity of human gastric juice for E. coli before and after adding vitamin B_{12}.



Effect of Pepsin followed by Chymotrypsin on binding activity of H.G.J. for E.coli before and after addition of Vitamin B_{12}

Figure 25. **Effect of Pepsin followed by Chymotrypsin on binding activity of human gastric juice for <u>E. coli</u> before and after adding vitamin B_{12}.**



Effect of Pepsin followed by Trypsin and Chymotrypsin on binding activity of H.G.J. for E.coli before and after addition of Vitamin B_{12}

Figure 26. **Effect of Pepsin followed by Trypsin and Chymotrypsin on binding activity of human gastric juice for <u>E. coli</u> before and after adding vitamin B_{12}.**

significant the lower the intrinsic factor concentration of the juice (Fig. 27).

In Fig. 28 are illustrated the results of crystalline pepsin on the juice of a patient with jejunal diverticulosis in whom the evidence for deficiency of vitamin B_{12} was marginal (Table 21). An interesting feature here was the loss of intrinsic factor concentration on incubation without enzymes (54.4 to 27.5 ngm./ml.). This was associated with very marked loss of intrinsic factor concentration after peptic digestion and the significant loss of binding activity, whether the juice is digested before or after the addition of vitamin B_{12}, is consequently not surprising. Some fall in the intrinsic factor concentration of gastric juice on incubation will have been noted in the results illustrated previously. Though variable, in no case was the fall as marked as it was in the juice from this patient with jejunal diverticulosis.

A better idea of the relevance of the experiments using crystalline enzymes to the situation <u>in vivo</u> can be obtained from two experiments, the results of which are illustrated in Figs 29 and 30. Here freshly obtained gastric juice was incubated at 37° C and at pH2. Native pepsin had not been inactivated and the juice was therefore digesting itself. Alkaline denaturation of pepsin was carried out on aliquots at zero time, at four and at six hours. Obviously with incubation there was significant loss of binding both of juice which had been autodigested before and also after the addition of vitamin B_{12}. The results are in fact similar to those obtained using

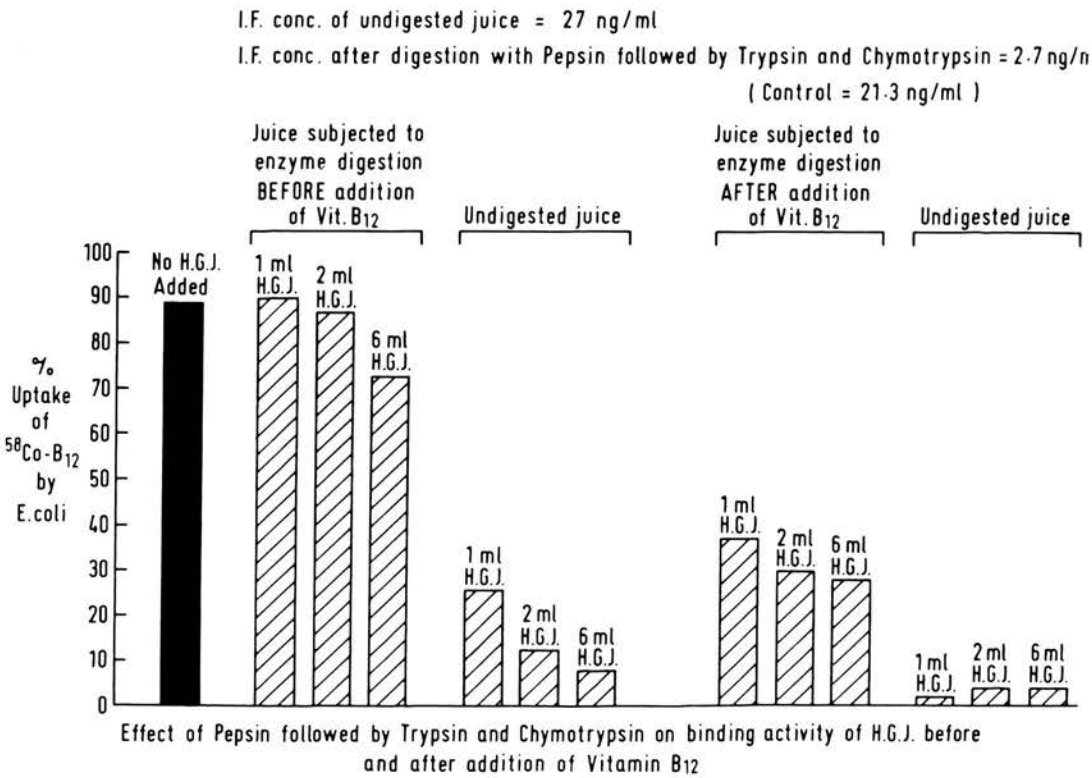


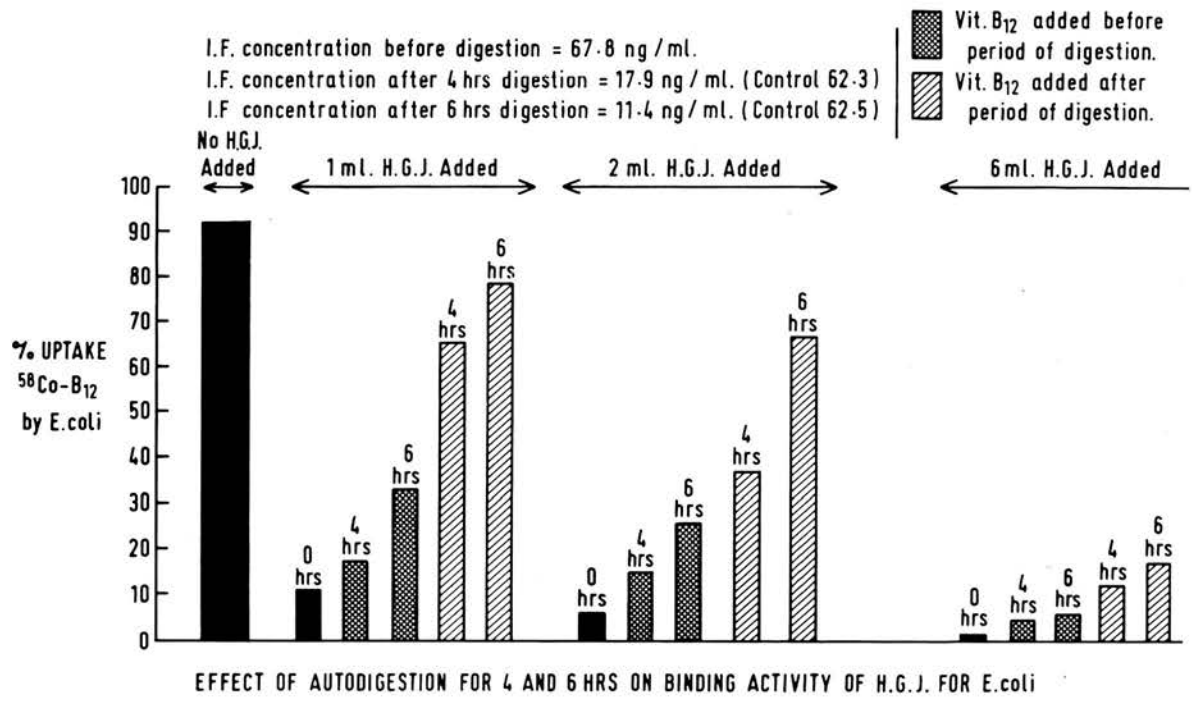
Figure 27. **Effect of Pepsin followed by Trypsin and Chymotrypsin on binding activity of human gastric juice for <u>E. coli</u> before and after adding vitamin B_{12}.**

TABLE 21

Haemoglobin	11.7 gm. per 100 ml.
Sternal marrow	Normoblastic
Serum vitamin B_{12} level	129 and 218 uugm. per ml.
Schilling Test	11.6% and 9.9% recovery
Schilling Test on 4th day of Tetracycline therapy	16.8% recovery
Figlu Test	Strongly positive
Serum folate	5.7 mugm. per ml.
Augmented Histamine Test	4.0 m.Eq. acid in post histamine hour containing 5633 ngm. of intrinsic factor

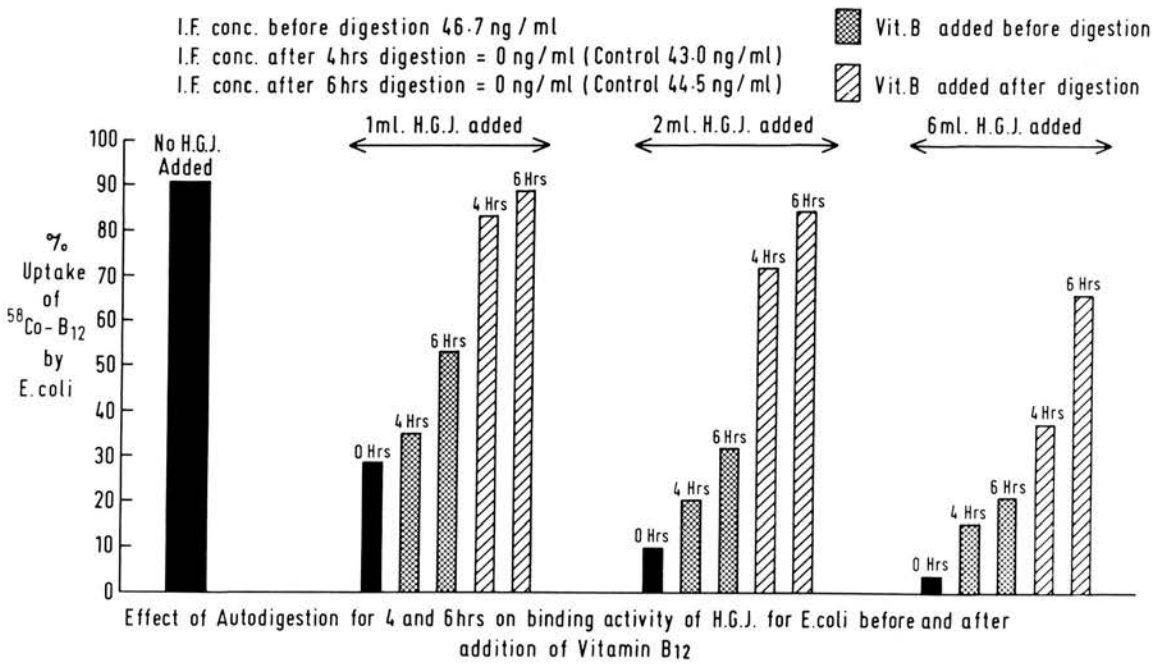
Results of studies in patient with jejunal diverticulosis

(see Fig. 28)



EFFECT OF AUTODIGESTION FOR 4 AND 6 HRS ON BINDING ACTIVITY OF H.G.J. FOR E.coli

Figure 29. **Effect of autodigestion for 4 and 6 hours on the binding activity of human gastric juice for E. coli before and after the addition of vitamin B_{12}.**



Effect of Autodigestion for 4 and 6hrs on binding activity of H.G.J. for E.coli before and after addition of Vitamin B_{12}

Figure 30. **Effect of autodigestion for 4 and 6 hours on the binding activity of human gastric juice for <u>E. coli</u> before and after the addition of vitamin B_{12}.**

crystalline enzymes and it is relevant to the subsequent discussion to note that here, too, falling intrinsic factor activity (Fig. 30) was accompanied by autodigestion having a very much more significant effect on the binding activity of the gastric juice.

DISCUSSION

Method of study

The most obvious way of studying the gastrointestinal flora is by examining the faeces but, as has been emphasised recently (Hayward, 1963), this not only does not reflect the flora of the small intestine but cannot even be assumed to be the flora of the whole of the large bowel. As might be expected the most obvious alternative is by studying gastrointestinal aspirates obtained by some form of intubation technique and there are many references to studies using such a method (Cregan and Hayward, 1953). In more recent years the Miller-Abbot tube has been popular, this method first being advocated for ileal aspiration by Nichols and Glen (1940) (Nadel and Gardner, 1956; Martini, Phear, Ruebner and Sherlock, 1957). Since the present work was initiated Shiner (1963) has described a capsule with which sterile samples may be obtained from the gastrointestinal tract.

There are obvious disadvantages to any intubation technique and these have been reviewed by various authors, in particular Cregan and Hayward (1953), Anderson and Langford (1958) and Donaldson (1964). It is difficult to be certain of the precise level at which one is

aspirating and this is aggravated by the fact that the small intestine may creep up along the tube. A very important problem is contamination of the tube during its passage through the nasopharynx or oropharynx. In an attempt to obviate this various authors have tried to seal off the aspirating segments of the tube with a gelatine capsule (Goldman, 1924), a rubber sheath (Nichols and Glen, 1940) or with colloidon (Thomson, Einhorn and Coleman, 1930; Nadel and Gardner, 1956), the seals being ruptured by pressure just prior to aspiration. It would seem unlikely that this precaution will totally or even significantly exclude contamination of gastrointestinal contents by organisms from higher up but it may reduce contamination of the interior of the tube, with perhaps consequent multiplication of microorganisms within this, prior to the time of aspiration. More relevant perhaps, particularly in some of the present studies, is that not only will saliva contaminate gastric contents but also, especially in patients with low gastric secretion in whom prolonged aspiration is often required, significant dilution may occur. Again, the presence of a tube in the gastrointestinal tract is hardly physiological and it is interesting to record that Anderson and Langford (1958) found a very abnormal flora in the upper small intestine after the tube had been in situ for 24 hours, though the initial aspirate was sterile. Though multiplication of organisms after the initial aspiration may have occurred within the tube, these authors suggest that disturbances of bowel motility consequent on the presence of the tube might have been contributing

to the abnormalities.

Because of the difficulties associated with intubation a different approach to the study of the gastrointestinal flora has been the direct sampling technique (Blacklock, Guthrie and Macpherson, 1937; Cregan and Hayward, 1953). This consists of injecting directly into a short segment of bowel at surgery a known volume of isotonic solution and culturing the fluid, as much of which as possible is sucked back. The problem with such a method is that it allows only a semi-quantitative study of the intestinal flora, that the gastrointestinal tract in the fasting anaesthetised patient is not necessarily the same as that in the ambulant patient and, of course, that it can only be carried out incidentally on patients who for one reason or another require abdominal surgery.

It is felt that the tube chosen for the present study has some advantages, the major one of which is its size. This results in minimal and often no discomfort whatsoever to the subjects, allowing them to partake of a normal ward diet after gastric juice is obtained. Though Anderson and Langford (1958) allowed the children they studied some soft food and milk, the fact that most studies have been done on fasting patients has been emphasised by French (1961). More recently Donaldson (1964) has also called for studies on non-fasting patients by mentioning how certain organisms will not grow using conventional culture methods but will do so using continuous flow culture techniques which might be expected to simulate more closely the situation in the gastrointestinal tract of a normal man.

It is also possible that the very marked pliability of the present tube at body temperature as well as its size, is perhaps less likely to interfere with small intestinal function and in particular with the "creeping" up the tube of the small bowel. It would therefore be expected that localisation of the site at which aspiration is taking place would be more accurate. Nonetheless the problem of contamination by oropharyngeal and nasopharyngeal flora remains and it is unlikely that the precautions taken, in particular the washing through of the tube after aspiration and after the aspirating segment left the stomach, totally prevented this. It is possible, however, that this procedure made multiplication of microorganisms in gastrointestinal contents trapped within the tube less likely. It is this problem of contamination in particular that, after the initial studies, resulted in the aspirates of the majority of the subjects being studied for faecal type organisms only.

In view of the different methods available to study the gastrointestinal flora one would like to know if the results of different methods used in the same patient are significantly different. There are few studies on this topic, probably because every method is rather laborious. However, results obtained on four patients were not significantly different whether samples were obtained by intubation or by direct aspiration at surgery (Anderson and Langford, 1958), and more recently Kalsar, Cohen, Artega, Yawn, Mayoral, Hoffert and Frazier (1966) have shown that the results obtained using an open-ended tube do not differ significantly from those obtained

using the Shiner capsule.

It will be obvious that no intubation procedure is likely to be blessed with complete success. In the present work the success rate varied from one group of patients to another. Thus the control subjects and patients who had undergone gastric surgery were easy to intubate with success. The more ill patients were, however, difficult and apart from delay or failure in getting the tube to leave the stomach, there was often delay in its passage down the small intestine with a particular tendency for the mercury bag to stick in the region of the junction of the second and third parts of the duodenum. Patients with cirrhosis of the liver were particularly difficult to intubate successfully, and the procedure failed in about half of the patients in whom it was attempted. Overall, however, seventy per cent. of intubations were successful. There were no complications associated with the procedure. In one patient the proximal end of the tube came loose and was swallowed. The tube was eventually passed as a coiled bundle of tube of rather alarming proportions. In one patient with cirrhosis studied in India, slightly blood-stained gastric juice was aspirated and the tube was immediately withdrawn with no untoward effects. Occasionally the mercury bag ruptured during the study with no noticeable effects.

As far as the methods used to culture and quantitate micro-organisms are concerned, these are based on well established bacteriological techniques. Kalsar and colleagues (1966) have emphasised the importance of getting aspirates diluted and on to the

culture media quickly if results are to be reliable and this was routine in the present study. Where one is dealing with samples containing such a potentially variable flora selective media are useful and of help particularly in inhibiting overgrowth by oropharyngeal flora, especially Streptococcus viridans and by coliform organisms where these are present. As far as the latter are concerned only minor interference with their identification occurred in that Neisseriae tend to grow, though poorly, on MacConkey agar plates. Difficulty was, however, occasionally encountered in the enumeration of Streptococcus faecalis, particularly when large numbers of coliform colonies were present. The method of culturing Bacteroides is not above criticism in that it was only during the latter part of the study that microaerophilic as distinct from strictly anaerobic conditions were used to try and isolate these organisms. It is unlikely, however, that this alone would result in these microorganisms to be missed entirely, particularly as the cultures were examined at intervals up to five days (Collee, personal communication). The greatest difficulty encountered was that these organisms are often to be found in the company of coliform organisms and growth of the latter is not entirely inhibited by the neomycin (70 ug./ml.) present in the blood agar plates. It is possible, therefore, that in the type of situation in which coliform organisms were present in large numbers, Bacteroides present in small numbers may have been missed.

Control subjects

The results in these patients show that the normal upper gastrointestinal tract is free from significant numbers of faecal type organisms. Though several authors, even in the early part of the century, considered this to be so, it was not accepted by all. Van de Reis (1922) using a type of cartridge and one of the pioneers in this field found a predominantly Gram-negative flora minaly in the ileum and Goldman (1924) considered Gram-negative bacilli, spore-bearing anaerobes and lactobacilli to be normal jejunal inhabitants. Bogendorfer (1922) considered that coliform bacilli were not normally present in the upper gastrointestinal tract, supporting the previous observations of Bessau and Bossert (1919). Thomson, Einhorn and Coleman (1930), however, again commented on the presence of Gram-negative "colon like" organisms in the jejunum. This confused situation led Cregan and Hayward (1953) to emphasise the inaccuracy of intubation as a method of studying the flora of the gastrointestinal tract. The present work, however, not only supports their own findings in the upper gastrointestinal tract but also the results of other intubation studies since (Anderson and Langford, 1958; Goldstein, Wirts and Kramer, 1963; Kalsar et al., 1966; Tabaqchali, Okobadejo, Neale and Booth, 1966). To quote from one of these only, the very detailed studies of Kalsar's group, very few jejunal cultures in 30 normal subjects contained significant numbers of organisms.

Escherichia were found on two occasions and lactobacilli were very unusual. It is quite possible that the major criticism of the earlier studies using intubation methods is not so much of the findings themselves but of the paucity of good methods with which to selectively culture organisms and to quantitate them.

An interesting facet of the present findings in normal subjects is the identical results obtained in the Europeans and the Indians studied. Donaldson (1964) has reviewed the evidence indicating that diet and environmental factors can affect the flora of the faeces in animals but the results obtained here would suggest that these are of less importance as far as faecal type flora in the stomach and small intestine is concerned, though this would have to be confirmed by the study of larger numbers. That a multiplicity of factors may affect the gastrointestinal flora is suggested by the results in the patients with miscellaneous disorders (Table 5, p. 32). The findings suggest that gastrointestinal disorders and perhaps steroids may result in abnormalities. Chemotherapeutic agents too were associated with abnormal findings. It is emphasised that the numbers studied are small but these types of situation are the subject of further study. There is much animal work mainly relating to the effect of antibiotics on the faecal flora of animals and of man and this has recently been reviewed by Donaldson (1964). The effect of these drugs on the small intestinal flora may be of great interest and in this context one might mention the well recognised development of refractoriness to antibiotic therapy in patients with symptoms related to abnormal

gastrointestinal proliferation of bacteria. The findings of Paulk and Farrar (1964) in the small intestine of a patient with jejunal diverticulosis after tetracycline therapy will be discussed later but the present author has found that in a patient with cirrhosis, antibiotic therapy has failed to eliminate an abnormal flora in the small intestine.

In this study observations on the bacteriology of the ileal contents were made on only six control subjects. Of these, one had coliform organisms present and another Clostridium welchii, both in significant concentrations. Cregan and Hayward (1953) found coliform organisms in this region in three of fourteen patients and in two of these they were present in significant numbers. Other authors (Martini et al., 1957; Anderson and Langford, 1958) report similar findings, namely, abnormal findings in the ileum in a small proportion of normal subjects, though in the group studied by Kalsar and colleagues (1966) the incidence of ileal abnormalities was higher. The finding of a faecal type flora in the ileum is therefore difficult to interpret and the demonstration of such a flora is likely to be of more significance the higher up the bowel that it is found. This, together with the fact that it can be particularly difficult to be certain of the exact location of the aspirating segment in the ileum, led to the majority of the studies in the present work being made at gastric and jejunal levels.

One final point which must be mentioned in this context concerns the definition of normality in the jejunum. More recent authors

have stated that the mere presence of faecal type organisms in the upper small intestine is abnormal (Tabaqchali and Booth, 1967). The author cannot agree with this since in a small proportion of the control subjects faecal type organisms have been found in small numbers though never in a concentration of 10^4 viable organisms per ml. or over. It is of interest to mention here that in the study of the urinary tract counts of 10^5 viable organisms per ml. or over in the urine have been suggested as abnormal (Kass, 1955), though more recently Effersøe and Jensen (1963) have suggested that the figure of 10^4 per ml. is more appropriate. Kalsar and his colleagues (1966) have reviewed previous findings, including the results of the present study, and in the light of their own result support the definition used here.

Patients with malabsorptive disease

These patients were investigated during the earlier part of the study by examining the jejunal and the ileal aspirates for as many organisms as possible. Bearing in mind the difficulties already discussed regarding the interpretation of an oropharyngeal type of bacterial flora, it is seen that in these patients the flora of the

small intestine does not differ significantly from those of the control subjects. In particular faecal flora is absent from the jejunum in most of the patients. Streptococcus faecalis appear in abnormal concentrations in the jejunum of two patients and, in addition, one of these patients had numbers of coliform organisms in the jejunum that were just abnormal. The findings in this particular patient should be interpreted with caution as he was on large dosages of corticosteroids. In view of the findings already discussed in two of three patients on these drugs it is possible that they might be responsible for the abnormal findings. In addition, this patient also had histamine-fast achlorhydria.

In the majority of these patients the flora in the ileal aspirate contained abnormal numbers of faecal type organisms. The difficulty of interpreting the results in this region have already been commented on but the findings suggest that the incidence of abnormality in this region in these patients may be greater than would be expected.

All except two patients had impaired absorption of vitamin B_{12} and all but one had steatorrhoea but it was impossible to draw any conclusions about the relationship of these and the finding of an abnormal flora. Certainly in patients Nos 39 and 40 broad spectrum antibiotics did not result in objective improvement of their malabsorption.

In the search for specific organisms as the cause of malabsorptive disease, it has been suggested that lactobacilli and yeasts might be responsible. It will be noticed that in the group of

patients investigated here these organisms were not infrequently isolated from the small bowel. It is not suggested, however, that this indicates that they have a causative role in this condition and in the low concentrations found they are very likely to be a secondary phenomenon, as has been previously suggested (French, 1961).

The necessity for a study such as the present on patients with malabsorptive disease who were not fasting has been stressed by both French (1961) and Donaldson (1964), particularly in the light of the improvement claimed to have occurred following antibiotic therapy in some of these patients. The present findings are, however, in accord with the observations of previous observers, which have failed to reveal any significant difference between the findings of patients with malabsorptive disease (Nadel and Gardner, 1956; Anderson and Langford, 1958) including tropical sprue (Milanes et al., 1946) and controls. In view of the problem mentioned about the difficulty of interpreting abnormalities of the ileal contents, and in the light of the knowledge that one is studying a disease which primarily and more severely affects the upper small bowel, it seems unlikely that the apparent ileal abnormalities noted here are of significance.

Patients with pernicious anaemia

The results in these patients indicate that so far as a faecal type flora is concerned there is no demonstrable difference between the findings in the majority of patients with pernicious anaemia and those of control subjects. In patient No. 55 (Table 7, p. 35) it is possible that the abnormal findings might be explained by the presence of the single diverticulum in the upper small intestine which was demonstrated radiologically. However, radiological examination of the small bowel in the other patients in whom an abnormal flora was found revealed no anatomical abnormalities which might explain the findings. It may be relevant to mention that two of the patients (Nos 51 and 59) with abnormal findings were in complete remission at the time of study. It seems, therefore, that in some patients with pernicious anaemia there is an increase of faecal type organisms in the jejunum. In the light of the findings in control subjects the results of the studies on the ileal contents cannot be said to differ significantly from those of controls.

In general it may be said that the present findings do not support the observations of previous authors on the bacteriological findings of the small intestine in this disease. Much of the literature on this subject has been reviewed in the introduction to this thesis. Although Davidson (1928) was unable to support the suggestion of Moench and her colleagues (1925) which was based on

observations of the faeces of patients with pernicious anaemia, that the small intestinal content of Clostridium welchii was abnormal in these patients, their own findings based on observations of the "gastroduodenal contents" supported those of previous workers. Consequently, although the view that the features of the disease are attributable to the absorption of a toxin from the alimentary tract is not of course now accepted, it is still a hypothesis that an abnormal small intestinal flora exists, particularly as regards faecal type organisms. The high pH of the gastric, and therefore the intestinal, contents is invoked as being responsible for this.

There are two recent studies on the subject, that of Sherwood, Goldstein, Haurani and Wirts (1964) and that of Gray and Shiner (1967). Both these studies were carried out on fasting patients. Gray and Shiner, using the Shiner capsule (1963), studied the gastric and jejunal contents of eight patients with pernicious anaemia. The findings are difficult to compare with those of the present study in that the numbers of organisms were assessed semiquantitatively. In only one of the stomachs of these patients, however, were faecal type organisms (Escherichia coli) present in "profuse" numbers. In the others faecal type organisms were present in "scanty" concentrations in three cases and were not detected in four. In the jejunum "scanty" coliform organisms were found in three patients. These findings, which are in keeping with the results of the present study, are in contrast to those of Sherwood et al. (1964). These authors studied nine patients. The results are again difficult to interpret

in that although quantitative studies were carried out on intestinal aspirates, faecal type flora was not studied separately from oropharyngeal flora. "The most abundant and most frequently recovered organism was an α -haemolytic Streptococcus. Gram-negative organisms such as Proteus, Aerobacter, E. coli and Pseudomonas species were also present to an excessive degree." It is of interest that in two patients the counts were within the range that the authors define as normal and in general they tended to be below the counts obtained in patients they studied with post-gastrectomy steatorrhoea. A pertinent criticism of this study is that jejunal aspirates were taken through a Miller Abbott tube after prior aspiration by the same tube at ileal level.

Taken together, therefore, recent work would suggest that in pernicious anaemia the content of faecal type organisms in the small intestine may be normal and even when abnormalities are found they are not of the order suggested by earlier work which was almost certainly the result of lack of quantitation. The higher incidence of normal results in the present study is possibly due to the fact that patients were not studied in the fasting state. Normal peristaltic activity may be of much greater importance in keeping the small bowel free from abnormal bacterial proliferation in the presence of gastric achlorhydria.

In the light of subsequent discussion on the relation of an abnormal gastrointestinal flora to vitamin B_{12} absorption and the influence of intrinsic factor in protecting vitamin B_{12} from

microorganisms, it is of interest to recall the work of Baker and Mollin (1955). These authors found that in patients with pernicious anaemia, different quantities of intrinsic factor were required to restore normal absorption of vitamin B_{12}. Again some patients with undoubted pernicious anaemia may have more than the usual maximum amount of intrinsic factor in the gastric juice (200 ngm. in the post-histamine hour) which is accepted as diagnostic of this condition (Shearman and Finlayson, personal communication). It is possible that these observations could result from the presence of a gastrointestinal flora which differed either quantitatively or qualitatively in different patients. A similar argument could explain the response to antibiotics that has occasionally been recorded in patients with pernicious anaemia (Lichtman, Ginsberg and Watson, 1950). Alterations in the intestinal flora, either spontaneously or due to unknown factors, might also explain the temporary remissions which are recorded in the older literature as occurring in this condition. The relation of the bacteriology of the intestinal tract in patients with pernicious anaemia and their gastric intrinsic factor output is the subject of further study.

Patients with blind or stagnant loops

As discussed in the introduction, there was good circumstantial evidence to relate the symptoms and signs associated with these disorders to the presence of an abnormal gastrointestinal flora. Very few studies, however, had actually been carried out on the gastrointestinal contents of such patients and those that were done were carried out on patients who had required surgery for their conditions.

From the present work it is apparent that an abnormal small intestinal bacteriology is a frequent finding in these patients and that this may be profuse. It is of interest that in the patients with impaired absorption of vitamin B_{12} which could be corrected by antibiotics and who had a surgically acquired stagnant loop in the distal small intestine (Table 10, p. 40), the flora was abnormal in the upper small intestine. Bishop (1963) has found an abnormal flora in the small intestine both proximal and distal to the site of surgically fashioned loops in dogs and the present findings suggest that the same may occur in man. In the other patients in whom absorption of vitamin B_{12} was normal, or if abnormal was unaffected by antibiotics, the flora was normal except in one case. This patient had subacute intestinal obstruction and the work of Bishop and Allcock (1960) suggests that this could explain the abnormal findings. It should be emphasised that in the patients with impaired absorption of vitamin B_{12} not due to the blind or stagnant

loop or to pernicious anaemia, the major reason for the impaired absorption was presumably bypassing of the ileum. In that the tests currently available to study vitamin B_{12} absorption do not quantitate absorption it is impossible, however, to exclude the possibility that the blind or stagnant loop was not also contributing to this.

In the patients with jejunal diverticulosis the flora was frequently of an abnormal faecal character in the absence of metabolic abnormality and though most of the patients had gastrointestinal symptoms it was not always possible to be certain that the diverticula were responsible for these. In addition, two patients had abnormal bacteriological findings (No. 78: Table 9, p. 38; and the patient with pernicious anaemia, No. 55: Table 7, p. 35), and in each of these only a single diverticulum was demonstrable radiologically. It would seem, therefore, that an abnormal flora does not by itself indicate that there are likely to be associated metabolic abnormalities. This may well be due to the fact that qualitative as well as quantitative abnormalities are important. This receives support from the observations of Goldstein, Cozzolino and Wirts (1963) of steatorrhoea associated with a single large duodenal diverticulum in a patient in whom an abnormal faecal type flora was demonstrable in the upper small intestine, and also from the observations of Paulk and Farrar (1964) in a patient with jejunal diverticulosis in whom, though vitamin B_{12} absorption was corrected by antibiotics, the flora after antibiotics was similar to that found prior to therapy. The most numerous organism Paulk and Farrar isolated from their patient

was a strain of Escherichia coli and though the uptake of vitamin B_{12} by this was similar before and after antibiotic treatment, its metabolic requirements were different. Rao, Tamhane and Sreenivasan (1962) have, however, recorded altered metabolic behaviour of Escherichia coli in relation to vitamin B_{12} after antibiotic therapy. This aspect of the findings will be discussed again in relation to the in vitro work. Meantime, from the qualitative point of view and in the light of subsequent discussions in patients who had undergone gastric surgery, it is interesting to note that two patients had steatorrhoea and Bacteroides were found in one of these.

Another reason why an abnormal flora might be present in the absence of symptoms or metabolic abnormality is because the method used is simply not a good indicator of the degree of intestinal contamination by these organisms. It is, for example, easy to imagine the aspirating segment of the tube lying at the neck of a single diverticulum. In this situation it is likely that measurement of the excretion of urinary indican might be a useful additional indicator of a quantitatively abnormal gastrointestinal flora since, in general, there appears to be a reasonably good correlation between the estimation of bacterial contamination as determined by intubation and the urinary excretion of indican (Tabaqchali et al., 1966). A third alternative is that factors quite apart from either the quantity or quality of the flora are important. Some of these in relation to vitamin B_{12} are gone into more fully in the discussion of the findings of the in vitro studies.

Patients with gastric surgery

Kinsella, Hennessy and George (1961) found heavy growth of bacteria in the afferent loop of patients with a partial gastrectomy undergoing surgery. Goldstein, Wirts and Kramer (1961) and Wirts and Goldstein (1963) studied the intestinal contents of similar patients quantitatively and recorded a high incidence of abnormal findings, and considered there was a relationship between abnormal bacterial proliferation and the presence, though not the degree, of steatorrhoea. The present work not only confirms that of the Goldstein group as far as the bacteriological findings are concerned but also establishes that similar findings occur in the gastrointestinal tract of patients after gastroenterostomy.

The method of studying the gastrointestinal flora of these patients employed in this study differs from that of Goldstein and his group in that they were aspirating directly from the afferent loop in as many cases as was possible and in that their patients were fasting. The present results do not suggest that the faecal fat excretion differs significantly between those patients with normal and abnormal bacteriological findings and this finding applies to all patients regardless of the type of operation. It could be that our definition of normality is either too strict or that bacterial counts must rise considerably above normal before steatorrhoea occurs. Tabaqchali and Booth (1966) have, for example, suggested that

patients with bacterial counts of more than 1 x 10^8 per ml. had more pronounced steatorrhoea. The stool fat content in the author's patients has been related to bacterial counts of 10^5/ml. and over, 10^6/ml. and over and 10^7/ml. and over without demonstrating any significant relationship. Once more it may be that qualitative rather than quantitative abnormalities of the bacterial flora are important. Bacteroides have, for example, been suggested as important in the causation of steatorrhoea, especially in view of their in vitro activity on bile salts (Drasar, Hill and Shiner, 1966), though Streptococcus faecalis and other bacterial strains can also do this (Portman, Shah, Antonis and Jorgensen, 1962). In the present work Bacteroides have been found in two cases with a partial gastrectomy, the daily stool fat of these cases being 5.2 and 4.6 gm.

The findings do not exclude the possibility that steatorrhoea can be caused by abnormal proliferation of faecal type organisms, though these results indicate that steatorrhoea can be absent even when the juices contain abnormal numbers of organisms. This is also in accord with the results recorded in patients with blind or stagnant loops. Steatorrhoea occurred, also, in some of the patients with gastric surgery in the absence of an abnormal bacterial flora and it is possible that inadequate mixing of food with digestive enzymes or impaired pancreatic function may be responsible for the abnormal fat loss in these cases (Wollaeger, 1950; Shingelton, Isley, Floyd, Sanders, Baylin, Postlethwait and Ruffin, 1957; Lundh, 1958; Tyor and Ruffin, 1958; Butler, 1960).

Diarrhoea after gastric surgery is another ill-understood phenomenon and the findings indicate that diarrhoea and steatorrhoea can exist independently of each other in both groups of patients. This supports the observations of Clark, Crooks, Dawson and Mitchell (1964) in post-gastrectomy patients. As is already recognised, the incidence of diarrhoea in the present patients was higher after gastroenterostomy with vagotomy than after partial gastrectomy; however, the bacteriological findings in the two groups were similar. Though only one patient after partial gastrectomy had diarrhoea, half of the patients with this type of operation had an abnormal flora. There is no support in the present findings for the suggestion, most recently voiced by Amendola (1965), that diarrhoea after gastric surgery may be related to abnormal bacterial contamination.

Failure to absorb vitamin B_{12} may follow partial gastrectomy and gastroenterostomy. This is usually due to lack of intrinsic factor and only rarely to a blind loop syndrome (Adams, 1958; Goldstein et al., 1961). Impaired absorption of the vitamin in these present patients was commoner after partial gastrectomy but the interval after operation was longer in these patients. It could always be corrected by intrinsic factor. Except in one case in which there was a little acid, achlorhydria was present in the patients with impaired absorption of vitamin B_{12}. However, only half of the patients in either group who had achlorhydria had impaired absorption of the vitamin, suggesting that loss of acid secretion precedes loss of intrinsic factor, as is usual also in pernicious anaemia.

The fact that impaired absorption of vitamin B_{12} was corrected by intrinsic factor in these cases makes it unlikely that organisms could be playing any significant role in vitamin B_{12} absorption even though this would be undetectable with present techniques. Nonetheless, by far the majority of patients with impaired absorption of vitamin B_{12} had an abnormal gastrointestinal flora particularly after gastroenterostomy and this relationship was statistically significant in the stomach after partial gastrectomy and in the stomach and jejunum after gastroenterostomy. These figures may, however, be influenced by the fact that patients with normal absorption of vitamin B_{12} are more likely to have free acid in the stomach and consequently, as has already been pointed out, they will tend to have a normal gastrointestinal flora. Because of this the relationship between vitamin B_{12} absorption and the bacteriological findings was reassessed in achlorhydric patients only (Table 16, p. 49). When this is done, though the figures again suggest a relationship between bacteriological abnormalities and impaired absorption of vitamin B_{12}, they are no longer statistically significant mainly because the residual numbers of patients are small. Tabaqchali and her colleagues (1966) have suggested that there is a relationship between the finding of abnormal numbers of Escherichia and impaired absorption of vitamin B_{12} in patients after gastric surgery as well as in other patients with blind or stagnant loops. Unlike the present group of patients, in several of the cases they studied after gastric surgery absorption of vitamin B_{12} was not corrected by intrinsic factor.

It is not clear from their work, however, whether gastric acidity was taken into consideration.

Patients with disorders of the liver

The findings in the patients with cirrhosis confirm those of Martini, Phear, Ruebner and Sherlock (1957) though in none of these patients was the flora profuse. Only one patient had portosystemic encephalopathy (No. 143, Table 17, p. 50) at the time of the study and though in this patient the flora was abnormal, so also was it in several others without encephalopathy. It is difficult to assess the significance of an abnormal small intestinal flora in these patients as their clinical state may be influenced by the degree of parenchymatous liver reserve as well as the size of the portosystemic shunt. Indeed, while accepting the importance of abnormal bacterial proliferation in the intestine of patients with certain liver disorders this situation is a good illustration of how the metabolic aberrations brought about by either quantitative or qualitative alterations of the flora are governed significantly by "extrafloral" factors. As has already been mentioned, it is likely that similar factors, at present not understood, operate in other situations in

which an abnormal flora may be associated with metabolic derangements.

Even when neurological symptoms and signs are present in a patient with cirrhosis of the liver the significance of an abnormal flora in the small intestine is not known. Though it has been suggested that the main source of the metabolites giving rise to encephalopathy in these patients is bacterial activity in the colon, it is possible that the abnormalities in the small intestine are significant (Martini et al., 1957; McDermott, 1967, including discussion). The various forms of colon bypass surgery suggested for this condition are based on the premise that either the main source of abnormal metabolites originate in the colon or that it is from the colon that the abnormal small intestinal flora giving rise to the metabolites originates. In this context it is relevant to mention that recolonisation of the ileum in some of these patients, who have usually undergone ileorectal anastomosis, is reported. The suggestion that the ileocaecal valve (McDermott, 1967) should be preserved to prevent this again presupposes that the lower bowel is the source of small intestinal contamination in these patients.

One can of course only speculate as to why an abnormal flora occurs in these patients. Low gastric acid secretion is usual in cirrhotic subjects and Scobie and Summerskill (1964) found five of the cirrhotic subjects they studied to have one or less milliequivalent of hydrochloric acid in the post-histamine hour after maximal histamine stimulation. In the present study it was found that the patient with encephalopathy had histamine-fast achlorhydria. In

view of the results already described in other patients with achlorhydria it is likely, however, that other factors are important. Patient No. 141, for example, had normal liver function tests and mild changes on biopsy of a considerably enlarged liver. Splenomegaly and oesophageal and gastric varices were present, and in the light of the subsequent discussion it is tempting to speculate whether impaired intestinal motility might result from venous congestion and thus enable abnormal bacterial proliferation to occur. The data available on the other patients are insufficient to carry this discussion further but the intestinal motility of these patients merits further study. Martini and his colleagues (1957) commented on the slow transit of their intestinal tube in these patients and this was the author's experience also. In fact, in a very considerable proportion of patients with cirrhosis the procedure had to be abandoned. Certainly in the light of present knowledge it cannot be concluded that the colon is the source of the small intestinal abnormalities found in some of these patients. This is discussed further in the subsequent section.

The mechanism of abnormal bacterial proliferation

To explain the occurrence of abnormal bacterial proliferation in some patients would require some knowledge of the mechanisms which normally keep the small intestine free from significant numbers of faecal type organisms. Gastric acidity has for long been credited as being of overriding importance in this respect. It is obvious that a low pH will inhibit the growth of bacteria and this is reflected in the fact that in the study of the patients who had undergone gastric surgery very few of those with free acid in the stomach after either operation had an abnormal flora in the gastrointestinal tract. However, about half of the patients who had developed achlorhydria ($pH > 6$) had normal bacteriological findings. The findings in the patients with pernicious anaemia and in the patients with histamine-fast achlorhydria also suggest that factors other than gastric acidity must operate to keep the small intestine free from abnormal bacterial proliferation. Cregan, Dunlop and Hayward (1953) first suggested this, an observation which has been supported by the work of Goldstein, Wirts and Josephs (1962).

The various factors which might be operating have been reviewed recently by Donaldson (1964) but normal peristaltic activity of the gastrointestinal tract would seem to be important. Dixon and Paulley (1963) found abnormal bacterial proliferation in the small intestine of animals with drug induced paralytic ileus and this was

the reason for trying the effect of propantheline bromide in subjects in the present study. In this context it is worth recalling the observations of Howie and his colleagues on the finding of Clostridia in the stomachs of patients who had recently undergone gastric surgery (Howie, Duncan and Mackie, 1953; Duncan, Goudie, Mackie and Howie, 1954). Certainly gastric surgery and the various congenital and acquired intestinal cul-de-sacs and loops studied here may well render peristaltic activity less effective as well as in themselves being the cause of gastrointestinal stagnation. Support for this concept comes from the abnormal bacteriological findings recently described in the small intestine of a patient with scleroderma who had steatorrhoea and impaired absorption of vitamin B_{12} (Salen, Goldstein and Wirts, 1966). In this condition and also in diabetic steatorrhoea in which disturbance of gastrointestinal motility is invoked as the cause of the syndrome, there may be a good response to antibiotic therapy (Malins and French, 1957; Sumi and Finlay, 1961; Khan, Jeffries and Sleisenger, 1965).

The source of an abnormal bacteriology in these patients is also a difficult problem. In addition to the work of Howie and his colleagues referred to above, Schwabacher, Salsbury and Loosemore (1959) found that a large proportion of patients undergoing gastrectomy grew coliform organisms in the gastric aspirate, sometimes within 2 - 4 hours of operation. They suggested that these organisms must come from the lower small bowel and colon and received support from Smiddy and Pratt (1960), though these latter workers

presumed a lower bowel source for organisms in the gastric remnant after gastric surgery, simply because coliform organisms were present. However, infants with complete intestinal obstruction from birth (Bishop and Anderson, 1960) and adults with small intestinal obstruction (Bishop and Allcock, 1960) develop a faecal type flora proximal to the site of obstruction and it seems likely, therefore, that we are constantly ingesting faecal type organisms in small numbers and that these remain undetectable until some change occurs in bowel function or bowel anatomy which enables them to multiply before they are moved on. French (1961) and Donaldson (1964) both emphasised the importance of examining the small bowel flora of patients who were not fasting in case shortage of nutrients was rendering organisms which would normally have been present in significant numbers undetectable. It seems more likely, in fact, that lack of food may give rise to abnormal numbers of organisms which would not be present in the normally eating individual simply through diminished gastrointestinal activity. The author feels this is likely to be the case particularly when that other factor in keeping the small intestine free from bacterial contamination, namely gastric acidity, is not operative, and feels this may largely explain the differences noted here in patients with pernicious anaemia as compared to the results of previous studies on the same patients.

The mechanism of abnormal vitamin B_{12} absorption in the blind or stagnant loop syndrome

This is an aspect of small intestinal bacteriology in which there is ever increasing interest. The main metabolic consequences attributed to an abnormal flora in the small intestine are steatorrhoea and impaired absorption of vitamin B_{12}. More recently, protein deficiency (Neale, Antcliff, Welbourn, Mollin and Booth, 1967) has been said to be due to bacterial degradation of dietary protein in the gastrointestinal tract.

The present work has described studies which attempt to elucidate the method by which impaired absorption of vitamin B_{12} results. There is no evidence at present that an abnormal flora affects vitamin B_{12} absorption through damage to the mucosal site of absorption, and though an abnormal flora could cause deficiency of vitamin B_{12} by producing some abnormal metabolite which could impair absorption of the vitamin, it seems that contact between the abnormal flora and the intestinal contents is necessary for this to take place (Donaldson, 1965). It is possible, therefore, that organisms in the intestine bind the vitamin and render it unavailable to the host (Halsted, Lewis and Gasster, 1956; Doig and Girdwood, 1960). The commonest organisms isolated in patients with an abnormal faecal flora belong to the group Enterobacteriaceae and it has been demonstrated that these are able to take up vitamin B_{12} very avidly. Under the con-

ditions of our experiments a one ml. overnight culture of Escherichia requires about five hours to attain its maximum uptake of vitamin B_{12} and food takes about four hours from the time of intake to reach the large bowel (Starling and Lovatt-Evans, 1962). In the small intestine, however, it is likely there is a constant supply of nutrients and continuous removal of metabolic products permitting continuous growth of microorganisms once these have become established. This type of situation is more likely to be reflected by the experiments using four hour growing cultures and consequently most of the in vitro work has employed this type of culture. In these circumstances it has been demonstrated that even small growing cultures can take up as much vitamin B_{12} as larger cultures within the space of an hour even in the presence of gastric juice (Fig. 15). It has also been demonstrated that much more vitamin B_{12} can be added to these cultures without saturating their capacity to take up the vitamin (Fig. 16). The author is uncertain exactly what amount of vitamin B_{12} will saturate the 10 ml. cultures except that the figure lies between 3/15 ugm. and 7/15 ugm. which, relatively speaking, are very large quantities of vitamin B_{12}.

The main objection to the concept of bacterial competition with the host for vitamin B_{12} is that it is known that gastric juice will bind cyanocobalamin, rendering it unavailable to microorganisms that would otherwise take it up in vitro (Ternberg and Eakin, 1949; Bird and Hoevet, 1951; Burkholder, 1952; Hoff-Jørgensen, 1952). Since under physiological conditions vitamin B_{12} is present in the small

intestine bound to intrinsic factor, it has been suggested that the vitamin is not likely to be available to microorganisms *in vivo* particularly as there is no known intraluminal mechanism in man capable of releasing vitamin B_{12} from its complex with intrinsic factor (Glass, 1963). Donaldson (1962), however, demonstrated that significant quantities of vitamin B_{12} were taken up by the contents of loops which had been created surgically in the intestine of rats even when this had been previously bound by gastric juice. More recently Tabaqchali and her colleagues (1966) have suggested that after partial gastrectomy organisms may depress absorption of vitamin B_{12} given with intrinsic factor and that the most likely organisms to be responsible for this activity are *Escherichia* since these are the commonest organisms isolated from these patients and from other patients with blind or stagnant loops. These observations are in agreement with those which have been made in the present work.

From the point of view of how *Escherichia* might bring about deficiency of vitamin B_{12} it is of interest to discuss the relevance of the in vitro work described further. The first problem is to consider to what extent these experiments represent the quantitative relationship between the vitamin B_{12} in food and the amount of intrinsic factor secreted. Any deductions in this context must be very speculative. From the work of Ardeman and Chanarin (1963), however, it is known that the mean intrinsic factor output in the post-histamine hour in normal subjects is 8900 ngm. Though the dietary intake of vitamin B_{12} is very variable (Bozian, Ferguson,

Heyssel, Meneely and Darby, 1963) it has been suggested that the minimal daily intake of vitamin B_{12} necessary to guarantee the physiological requirements of the vitamin is of the order of 4 to 10 ugm. daily (Grässbeck, 1959b; Heinrich, 1964). Assuming an intake of 5 ugm. in a meal it would seem that of these experiments (in which 1/15 ugm. of vitamin B_{12} was brought into contact with gastric juice containing just over 70 ngm. per ml. of intrinsic factor) those using 1 - 2 ml. of gastric juice are the most likely to mirror the situation in vivo in an average man after an average meal.

From the present work it can be said that even large quantities of intrinsic factor cannot bind vitamin B_{12} to which organisms have obtained first access. After partial gastrectomy and gastroenterostomy with vagotomy an abnormal faecal type flora in the stomach or stomach remnant is not uncommon and it has been shown that some patients with blind or stagnant loops, even when these are situated in the distal small intestine, may have an abnormal flora not only in the upper small intestine but in the stomach. It is possible, therefore, that in such circumstances organisms may obtain first access to vitamin B_{12} but in view of the rapidity with which intrinsic factor binds the vitamin the uptake by microorganisms is not likely to be very significant. However, uptake even of vitamin B_{12} which has been bound to intrinsic factor appears to proceed if organisms continue to grow (Figs 17 and 18: Table 20). It should be emphasised here that the optical density measurements in Table 20, though indicating that significant growth has occurred, also shows that this

has not been very marked. In fact the medium added as a source of extra nutrients, i.e. medium used to assay vitamin B_{12} microbiologically using Lactobacillus leichmannii, supported growth of the organisms used in the present study very poorly indeed and it is very likely that the results of the experiments illustrated in Figs 17 and 18 have considerably underestimated the effect of growth in enabling uptake of vitamin B_{12} that was previously bound by gastric juice. It could be argued that the observations made are the result of loss of intrinsic factor activity during the period of incubation. This is not, however, very likely. Intrinsic factor activity is very stable after it has come into contact with vitamin B_{12}. Though in some of the in vitro work involving the use of enzymes there is loss of binding activity in specimens of gastric juice incubated as controls, study of the results also indicates the greater binding activity of control samples of juice to which vitamin B_{12} was added prior to the period of incubation. The recent observations of Abels and Schilling (1964) are of interest in this context.

Growing cultures of Escherichia can take up large amounts of vitamin B_{12} quickly. The present work suggests that growth is more important than numbers in enabling organisms to take up vitamin B_{12} though, in that the former gives rise to the latter by multiplication it is difficult to distinguish the two. In this context the work of Booth and Heath (1962) is of interest. They found that live Escherichia took up similar quantities of vitamin B_{12} to organisms which had been killed by heating for four hours at 56° C. The fact that the live organisms consisted of the precipitates of cultures resus-

pended in saline, that is significant growth was not likely, is a possible explanation for these findings. The present work indicates that heating a growing culture for ten minutes at 56° C will significantly reduce its uptake of vitamin B_{12}. Growth of organisms is likely to be a continuous process in the gastrointestinal tract where nutrients will be constantly supplied, toxic products removed and pH levels suitable for vitamin B_{12} uptake maintained. The presence of gastrointestinal stagnation and the relatively wide pH range over which uptake of vitamin B_{12} can occur will obviously be relevant in this context as indeed also will be the demonstrated avidity of growing organisms for the vitamin even after considerable dilution. It has, however, not been possible to confirm the findings of Rao and his colleagues (1962) that vitamin B_{12} uptake by Escherichia increases in hyperosmolar solution.

It is obvious that the less the secretion of intrinsic factor the less is the binding activity of the gastric juice and the greater the uptake of vitamin B_{12} by Escherichia. Thus low levels of secretion which might normally enable the patient to absorb the minimal physiological requirements of vitamin B_{12} may not be able to do so in the presence of microorganisms. Low levels of gastric secretion are commonly reported in patients with jejunal diverticulosis, gastric anacidity being not uncommon (Crawford and Freeman, 1961; Cooke et al., 1963). This suggests that the intrinsic factor output in these patients may be reduced. There is very little factual information on this subject though some early observations have suggested

that this may be so (Castle, Heath and Strauss, 1931; Schlesinger, 1933; Verloop and Florjn, 1951). More recently Ardeman and Chanarin (1965) report a case of jejunal diverticulosis and vitamin B_{12} deficiency with 1000 ngm. of intrinsic factor in the post-histamine hour. A patient studied by the author with a blind loop syndrome following gastroenterostomy and vagotomy had 873 ngm. The intrinsic factor output in the patient with jejunal diverticulosis and marginal absorption of vitamin B_{12} whose juice was used in this study was 5633 ngm. in the post-histamine hour.

Most of the previous work on the effect of enzymes on the binding activity of gastric juice has been carried out by seeing how much bound vitamin is rendered dialysable after exposure to the enzymes (Glass, 1963). Experimental details have been incomplete and no attempt seems to have been made to quantitate the effects of enzymes either in terms of binding activity or, of course, in terms of intrinsic factor concentration for the measurement of which methods have only become available recently. On the whole the present work using the bacterial uptake supports the previous findings. The effect of trypsin and chymotrypsin would not appear to be very significant but the effect of pepsin on the binding activity of gastric juice is likely to be very important even when the juice has had prior access to vitamin B_{12}. The intrinsic factor measurements indicate that the loss of binding is the result of diminished intrinsic factor concentration. In that the technique used to measure intrinsic factor enabled the measurements to be made only on juice to

which labelled vitamin B_{12} had not been added, it could be argued that the loss of binding of the juice exposed to vitamin B_{12} before digestion might be due to the effect of enzymes on vitamin B_{12} binders other than intrinsic factor. This is unlikely, however, since Ardeman and Chanarin (1965) have shown that 86% of the binding activity of gastric juice is due to intrinsic factor. Low levels of intrinsic factor secretion will make the digestive effect of pepsin on binding activity more significant. This will also depend on the extent to which falling secretion of intrinsic factor is accompanied by diminished pepsin secretion. The results of the experiments illustrated in Figs 29 and 30 would suggest that in certain patients a greater fall in intrinsic factor concentration relative to the concentration of secreted pepsin may well be an important factor. In the patients with jejunal diverticulosis the loss of intrinsic factor activity after incubation for four hours, even though no enzymes were added (Fig. 28), is higher than any encountered in the other gastric juices studied. Whether this is a coincidence or a feature which is particular to the gastric juices of these patients is uncertain. In an attempt to simulate conditions in these patients the rate at which gastric juice which has had its pepsin inactivated loses intrinsic factor activity in the presence of water and in the presence of supernatant taken from an Escherichia culture have been compared but no significant differences have yet been found. The author has not as yet had the opportunity of studying gastric juice from other patients with jejunal diverticulosis.

The activity of pepsin disappears rapidly with rising pH. Nonetheless 70% of the maximal peptic activity is still present at pH 4.5 (Farrar and Bower, 1967). The fact that as Escherichia grow they tend to produce an acid environment may therefore be important in prolonging the digestive activity of this enzyme. Quite apart from this it will be recalled that low pH seems to be unfavourable for absorption of the intrinsic factor-vitamin B_{12} complex at the intestinal phase (Herbert and Castle, 1961).

The above discussion has considered the quantitative relationship of this in vitro work to the in vivo situation in terms of intrinsic factor and dietary vitamin B_{12} only. A similar calculation suggests that between 500 and 1000 ml. of growing culture would represent the 10 ml. cultures in the situation in vivo, though in the light of the experiments with the growing 5 ml. cultures (Fig. 15) and those showing the avidity of the growing cultures for vitamin B_{12} (Fig. 16) this may be a considerable overestimate. The volume of the gastrointestinal contents in patients with blind loops is likely to be greater than 500 - 1000 ml.

Though great care must be exercised in the application of these observations in vitro to what occurs in the body, they do on the whole lend support to the concept that in certain situations Escherichia are capable of depriving man of vitamin B_{12} by direct competition. That this is not likely to be a property particular to Escherichia is suggested by the fact that most of the coliform organisms isolated from the gastrointestinal tract in this work take up vitamin B_{12}

similarly. In addition, some other organisms of the Enterobacteriaceae group, amongst them Alkalescens-Dispar and Citrobacter, also tend to produce an acid environment as they grow.

The major obstacle to this concept of the causation of vitamin B_{12} deficiency in patients with blind or stagnant loops is the well known observation that in such patients intrinsic factor does not correct impaired absorption of vitamin B_{12}. This is not the experience of all observers (Badenoch et al., 1955; Scudamore, Hagedorn, Wollaeger and Owen, 1958). One problem is that with the methods currently available to measure the absorption of vitamin B_{12} it is impossible to quantitate the effects of intrinsic factor on this. From the present work it also seems likely that the effect of microorganisms has also been greatly underestimated. This is the result of thinking of them as a culture in a test tube rather than as a mass of organisms situated in an environment which enables them to grow continuously. Certainly the findings in the patient with jejunal diverticulosis and marginally impaired absorption of vitamin B_{12} suggest that quite large volumes of gastric juice may be required to protect vitamin B_{12} from microorganisms at least in some patients. Again for obvious reasons, tests of vitamin B_{12} absorption in the presence of intrinsic factor usually employ sources of intrinsic factor other than human gastric juice. These may behave differently in vivo and certainly there is some evidence that their behaviour on exposure to enzymes is different (Reizenstein, 1959).

SUMMARY AND CONCLUSIONS

Using a fine polyvinyl tube to aspirate gastrointestinal contents, the bacterial flora in the gastrointestinal tract has been studied in the following patients:

1. A control group of patients, some European (15) and some Indian (11). A group of patients on various drugs was also studied (10).
2. A group of patients with primary malabsorptive disease (8).
3. A group of patients with pernicious anaemia and achlorhydria (including one patient with very low acid secretion) (24).
4. A group of patients with blind or stagnant loops in the small intestine and of patients with jejunal diverticulosis (22).
5. A group of patients who had undergone partial gastrectomy (26) and gastroenterostomy and vagotomy (21).
6. A group of patients with various disorders of the liver (15).

The finding of faecal type organisms in the normal small intestine was unusual. In those patients in whom they were present the concentrations found were very low. In the light of the findings in the normal subjects a concentration of faecal type organisms, as

represented by the group Enterobacteriaceae or Streptococcus faecalis, of 10^4 viable organisms per ml. aspirate or greater in the stomach or the jejunum is considered to be abnormal.

In patients with primary malabsorptive disease the bacteriological findings did not differ from those of the control subjects. The bacterial flora was frequently abnormal in patients with surgically or otherwise acquired anatomical abnormalities of the gastrointestinal tract and in patients with cirrhosis of the liver. In patients with achlorhydria some have an abnormal flora in the gastrointestinal tract but the incidence of abnormality in these patients is not of the order suggested by the older literature. The finding of an abnormal flora should take into consideration possible drug therapy.

Study of the findings in those patients in which they were abnormal indicates that abnormalities are not uncommon in the absence of metabolic derangements. In all patients with an ileotransverse colostomy, a loop in the distal small bowel and with jejunal diverticula an abnormal flora was always present when metabolic derangements, mainly impaired absorption of vitamin B_{12} in this study, occurred. An abnormal flora might, however, be present in the absence of such metabolic disturbances. This appeared to apply also to patients who had undergone partial gastrectomy and gastroenterostomy though there was a suggestion of a relationship between impaired vitamin B_{12} absorption and the presence of an abnormal flora which was difficult to interpret because of the influence of gastric secretion. The reason for this may be that qualitative variation in

the flora is more important than the essentially quantitative abnormalities demonstrated or that the method used is not a sufficiently good indicator of quantitative abnormalities. On the other hand, it is likely that factors other than the mere presence of a flora which is either quantitatively or qualitatively abnormal are important. These are, for example, obvious in patients with cirrhosis of the liver.

No evidence which will incriminate any single organism as a causal factor in the production of various symptoms and signs has been produced. Certainly it has not been possible to substantiate the theory that Bacteroides are responsible for steatorrhoea in the blind or stagnant loop syndrome but it must be emphasised that the incidence of steatorrhoea in our patients with an ileotransverse colostomy or jejunal diverticula was extremely low and the findings after gastric surgery are difficult to interpret because, as discussed, the steatorrhoea in these patients is likely to be of a mixed aetiology. As far as vitamin B_{12} absorption is concerned it would seem that several microorganisms can take this up in vitro. The avidity of Enterobacteriaceae for this is, however, striking and where an abnormal flora was detected in the gastrointestinal tract Escherichia were particularly common. The results of the studies in vitro suggest that these organisms may well be able to deprive their host of vitamin B_{12} by direct competition and, furthermore, highlight some of the factors in relation to vitamin B_{12} absorption, other than quantitative or qualitative abnormalities of the intestinal flora,

which may be important in this respect.

Growth and numbers of organisms are both important, the former possibly more so than the latter. Continued growth of organisms will enable them to take up vitamin $_{12}$ which has been bound by gastric juice and the lower the secretion of intrinsic factor the higher it is likely that the amount of vitamin B_{12} taken up by microorganisms will be. The activity of pepsin may have an important part to play in this respect as this enzyme can significantly affect the binding of gastric juice by vitamin B_{12} even if exposure of the juice to the enzyme takes place after it has come in contact with vitamin B_{12}. Important in this respect also, and possibly variable from patient to patient, will be the ratio of peptic to intrinsic factor secretion in the gastric juice.

The mechanism by which an abnormal flora becomes established is discussed. The findings in the patients who had undergone gastric surgery indicate that an abnormal flora in the presence of gastric acidity is unusual. However, in these patients as well as in those with pernicious anaemia and histamine-fast achlorhydria, the presence of abnormality was by no means invariable suggesting that some other method of keeping the intestinal tract free from bacterial contamination may be important. It is suggested that peristaltic activity is important in this respect and consequently that a better idea of the state of the intestinal flora in relation to normal findings is to be obtained from the study of patients who have not been fasting. Anatomical abnormalities in the gastrointestinal tract quite apart

from their mere presence, may give rise to abnormal bacteriological findings by interfering with the efficiency of normal peristalsis.

APPENDIX I

Materials and Methods

Media employed

1. DIFCO microinoculum broth

The material is reconstituted according to the maker's instructions. The final pH of the medium is 6.8 (DIFCO Manual, 1953). In some of the work double strength medium was used.

The vitamin B_{12} content of a large batch of this was determined using Lactobacilli leichmannii as the test organism

Vitamin B_{12} concentration = 550 uugm./ml.

In the present work 1/15 ugm. of radioactively labelled vitamin B_{12} was added per 10 ml. tube of broth. This is approximately twelve times as much vitamin as is already present in the tube.

2. Blood agar. This is made up by adding 5% sheep's blood to DIFCO Bacto agar (B140).

3. MacConkey agar. DIFCO (B75).

4. Rososa agar. OXOID P.M.221 (OXOID, 1956).

5. Lactose-egg-yolk-milk-agar medium (Willis and Hobbs, 1959)

Basal medium	400 ml. meat infusion broth (pH7)
Agar	4.8 G.
Lactose	4.8 G.
1% neutral red	1.3 ml.

The mixture is autoclaved at 15 lb./sq. in. pressure for 20 minutes. When this agar base has cooled to 50 - 55° C, 15 ml. of egg-yolk suspension and 60 mg. of sterile stock milk (prepared by autoclaving ordinary milk after removal of the cream by centrifugation) are added and the plates poured immediately.

6. Thallus acetate agar plates (Barnes, 1956)

For 100 ml. of medium

Peptone	1 G.
Lab. lemco	1 G.
Glucose	1 G.
Thallus acetate	0.1 G.

The peptone, lab. lemco and thallus acetate are autoclaved at 15 lb./sq. in. pressure for 20 minutes. The glucose is added as 5 ml. of a 20% solution immediately before the plates are poured.

7. Neomycin blood agar (Smith and Crabb, 1961)

This is blood agar with neomycin sulphate added in a concentration of 70 ug./ml.

(The author is indebted to the Preparation Room, University of Edinburgh Department of Bacteriology, for assistance in the preparation of Willis and Hobbs' medium and thallus acetate agar medium).

Biochemical differentiation of Enterobacteriaceae

The main reactions employed are illustrated in Table 22. In performing these the author made use of the various media made available in bijou bottles by Oxoid Laboratories Ltd. These include peptone-water sugars, glucose phosphate peptone water, Voges-Proskauer and Methyl Red medium and urea agar. Other necessary media were obtained from the Bacteriology Department, University of Edinburgh.

Method of dilution of ^{58}Co-vitamin B_{12}

Ampoules, as obtained from the Amersham Radiochemical Centre, contain 3.6 ugm., the activity of which is 10 uc. 6.4 ugm. of stable vitamin B_{12} were added to this and the volume made up to 15 ml. with sterile water.

Therefore 15 ml. volume contains 10 ugm. vitamin B_{12} with activity 10 uc.

and 0.1 ml. contains 1/15 ugm. vitamin B_{12} with activity 1/15 uc.

TABLE 22

	Gas from Glucose	Ferment Lactose	Ferment Sucrose	Production of Indole	Production of H_2S	Growth on Kosers Medium	Voges Proskauer Reaction	Methyl Red Reaction	Production of Urea	Motility
Escherichia θ	+	+	V	+	-	-	-	+	-	+
Citrobacter	+	+	V	-	+	+	-	+	-	+
Klebsiella	+	+	+	-	-	+	+	-	+	-
Cloaca	+	V	+	-	-	+	+	-	V	+
Hafnia	+	-	V	-	+	+	V	V	-	+
Proteus	+	-	V	V	V	V	-	V	+	+
Providencia	V	-	V	+	-	+	-	+	-	+
Salmonella	+	-	-	-	+	+	-	+	-	+
Arizona	+	V	-	-	+	+	-	+	-	+
Shigella	-	-*	V	V	-	-	-	+	-	-

θ Includes Alkalescens-Dispar - nonmotile, non-gasproducing, non-lactose fermenting variables of Escherichia

V - Variable

* - Sh. sonnei late

Bacterial uptake experiments

The following is an outline of the experimental details and the results obtained by the studies *in vitro*. In general sterile materials were used throughout. In all the experiments the sterility of the media and solutions employed was checked by setting up sterility controls - usually a 1 ml. aliquot in 10 ml. broth - in parallel with the appropriate experiment. Osmolality was measured using a Fiske Osmometer G.66 with the technical assistance of Mr. John Cowie, Department of Therapeutics. pH adjustments were made using a Beckman 72 pH Meter. To maintain sterility the electrodes were washed with sterile normal saline.

Details of the Rate of Uptake experiments. Method applies to experiments, the results of which are illustrated in Figs 3 - 6.

Take duplicate tubes containing 10 ml. broth A, A; B, B; C, C; D, D; E, E; F, F; G, G each containing 1/15 ug. ^{58}Co-vitamin B_{12}.

Inoculate A - F each at zero time with 1 ml. overnight culture of organisms being tested.

To G add 1 ml. of sterile normal saline.

At zero time centrifuge A, A at 3000 revs/min. for ½ hr

Incubate other tubes at 37° C.

At zero time + 1 hour centrifuge B, B " " " " "

" " " + 2 hours " C, C " " " " "

" " " + 5 hours " D, D " " " " "

" " " + 12 hours " E, E " " " " "

" " " + 24 hours " F, F " " " " "

In each case decant the supernatant and keep at -20° C.

Measure radioactivity in 5 ml. of the supernatant of each sample and compare it with the radioactivity in 5 ml. of the uninoculated standards G, G.

Rate of uptake of a 4 hr culture compared to rate of uptake of a 1 ml. overnight culture (Fig. 7).

(i) Take duplicate tubes containing 10 ml. broth A, A; B, B; C, C; D, D; E, E; F, F; G, G.

Inoculate A - F with 1 ml. overnight culture.

To G add 1 ml. of sterile normal saline.

Incubate at 37° C.

After exactly four hours add each tube to 1 ml. sterile saline containing 1/15 ug. ^{58}Co-vitamin B_{12}.

At zero time centrifuge A, A at 3000 revs/min. for ½ hr

Incubate other tubes at 37° C.

At zero time + 1 hour centrifuge B, B " " " " "

" " " + 2 hours " C, C " " " " "

" " " + 5 hours " D, D " " " " "

" " " + 12 hours " E, E " " " " "

" " " + 24 hours " F, F " " " " "

In each case decant supernatant and keep at -20° C.

(ii) As in (i) but cultures are not incubated for four hours prior to

being added to ^{58}Co-vitamin B_{12} in 1 ml. of sterile normal saline.

Measure the radioactivity in 5 ml. of the supernatant of each sample and compare it with the radioactivity in 5 ml. of the uninoculated standards G, G.

Results

		% Uptake		Mean
(i)	Tube A, A	43	43	43
	B, B	82	80	81
	C, C	85	82	84
	D, D	86	82	84
	E, E	80	72	76
	F, F	83	83	83
(ii)	Tube A, A	5	3	4
	B, B	15	12	14
	C, C	50	62	56
	D, D	78	74	76
	E, E	85	82	84
	F, F	80	80	80

Uptake of growing culture of E. coli after 1 hr in the presence of various quantities of human gastric juice (H.G.J.) (Fig. 8).

Take duplicate tubes containing 10 ml. broth A, A; B, B; C, C; D, D; E, E; F, F.

Inoculate A - E with 1 ml. overnight culture.

To F add 1 ml. sterile normal saline.

Incubate at 37° C.

After exactly four hours add:

A, A to 10 ml. sterile normal saline containing 1/15 ug. ^{58}Co-vitamin B_{12}.

B, B to 9 ml. sterile normal saline + 1 ml. H.G.J. containing 1/15 ug. ^{58}Co-vitamin B_{12}.

C, C to 8 ml. sterile normal saline + 2 ml. H.G.J. containing 1/15 ug. ^{58}Co-vitamin B_{12}.

D, D to 4 ml. sterile normal saline + 6 ml. H.G.J. containing 1/15 ug. ^{58}Co-vitamin B_{12}.

E, E to 10 ml. H.G.J. containing 1/15 ug. ^{58}Co-vitamin B_{12}.

F, F to 10 ml. sterile normal saline containing 1/15 ug. ^{58}Co-vitamin B_{12}.

Incubate for further hour at 37° C.

Centrifuge samples at 3000 revs/min. for ½ hr.

In each case decant supernatant and keep at -20° C.

Measure radioactivity in 5 ml. supernatant and compare with the radioactivity of the uninoculated standards F, F.

Results

	% Uptake		Mean
Tube A, A	84	82	83
B, B	21	18	20
C, C	17	10	14
D, D	6	2	4
E, E	4	4	4

Uptake by E. coli in hypertonic broth (Fig. 9).

Hypertonic broth was prepared by making up double strength DIFCO broth and adding equal proportion of 1.2 M phosphate buffer at pH 6.8.

Take duplicate tubes containing 10 ml. broth A, A; B, B.
Take duplicate tubes containing 10 ml. hypertonic broth C, C; D, D.
Take duplicate tubes containing 10 ml. broth E, E.

To all tubes add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water.

To A and C add 1 ml. sterile normal saline.
To B and D add 1 ml. H.G.J.

Inoculate A - D with 1 ml. overnight culture.
To E add 2 ml. sterile normal saline.
Incubate all tubes for six hours at 37° C.

Centrifuge samples at 3000 revs/min. for ½ hr.
In each case decant supernatant and keep at -20° C.

Measure the radioactivity in 5 ml. of supernatant and compare with radioactivity of 5 ml. of uninoculated standards E, E.

Results

	% Uptake		Mean
Tube A, A	90	90	90
B, B	26	29	28
C, C	90	90	90
D, D	26	20	23

To determine if a growing culture of E. coli can compete with gastric juice for vitamin B_{12} (Fig. 10).

(i) Take tubes A_1, A_2, A_3, A_4 containing 10 ml. broth.
Inoculate with 1 ml. overnight culture.

To A_1 and A_2 add 1 ml. H.G.J. at 37° C. and incubate for 4 hr.
Incubate A_3 and A_4 for 4 hr but add 1 ml. H.G.J. at 37° C. after 3¾ hr.

At the end of 4 hr add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water to A_1 - A_4.
Incubate further hour at 37° C.

(ii) Take tubes B_1, B_2, B_3, B_4 containing 10 ml. broth.
Inoculate with 1 ml. overnight culture.
Incubate at 37° C.

After 4 hours:
To B_1 and B_2 add 1 ml. of H.G.J. which has been incubated for 4 hr at 37° C. (Just prior to addition add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water to H.G.J.).

To B_3 and B_4 add 1 ml. of H.G.J. which has been incubated for ¼ hr at 37° C. (Just prior to addition add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water to H.G.J.).

Incubate further hour at 37° C.

Centrifuge at 3000 revs/min. for ½ hr.

In each case decant supernatant and keep at -20° C.

Measure the radioactivity in 5 ml. of supernatant and compare with radioactivity of 5 ml. of uninoculated standard (10 ml. broth + 2 ml. saline + 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water).

Results

	% Uptake	Mean
Tubes A_1, A_2	36 44	40
A_3, A_4	34 36	35
B_1, B_2	50 50	50
B_3, B_4	36 35	36

Effect of 1 ml. H.G.J. added at different times to a 1 ml. inoculum of E. coli in 10 ml. broth .

Effect of 1 ml. H.G.J. added at different times to a growing culture of E. coli (Fig. 11).

(i) Take tubes A, A; B, B; C, C; D, D; E, E; F, F; G, G; H, H each containing 1/15 ug. ^{58}Co-vitamin B_{12} in 10 ml. broth.

Inoculate A - F with 1 ml. overnight culture.

To H add 2 ml. sterile normal saline.

To A add 1 ml. sterile normal saline.

To B add 1 ml. H.G.J.

Incubate all tubes at 37° C.

After 15 minutes add 1 ml. H.G.J. to C.

After 30 minutes add 1 ml. H.G.J. to D.

After 1 hour add 1 ml. H.G.J. to E.

After 2 hours add 1 ml. H.G.J. to F.

After 6 hours add 1 ml. H.G.J. to G.

All additions to be made at 37° C.

Continue incubation at 37° C for 10 hr from zero time.

Centrifuge 3000 revs/min. and measure uptake in the usual manner using H as standard.

(ii) Take tubes A, A; B, B; C, C; D, D; E, E; F, F; G, G each containing 10 ml. broth.

Inoculate A - F with 1 ml. overnight culture and incubate at 37° C.

To G add 2 ml. sterile saline.

At the end of 4 hours add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water to each tube.

To A add 1 ml. H_2O.

To B add 1 ml. H.G.J.

Incubate all tubes at 37° C.

After 15 minutes add 1 ml. H.G.J. to C.

After 30 minutes add 1 ml. H.G.J. to D.

After 1 hour add 1 ml. H.G.J. to E.

After 6 hours add 1 ml. H.G.J. to G.

All additions at 37° C.

Continue incubation at 37° C for 10 hours from zero time.

Measure uptake as before.

Results

		% Uptake		Mean
(i)	Tube A, A	83	79	81
	B, B	5	8	7
	C, C	4	7	6
	D, D	9	6	8
	E, E	18	18	18
	F, F	64	57	61
	G, G	82	80	81
(ii)	Tube A, A	89	90	90
	B, B	10	7	9
	C, C	34	47	41
	D, D	53	59	56
	E, E	84	82	83
	F, F	88	83	86

Effect of adding H.G.J. at 2 and 6 hours to a 1 ml. inoculum of *E. coli* in 10 ml. broth (Fig. 12).

The format of this experiment was similar to part (i) of that just described (see p. 123).

Tubes taken A, A; B, B; C, C; D, D; E, E; F, F; G, G; H, H; I, I.

Tubes A - no gastric juice was added.

B - 10 ml. gastric juice added at zero time.

C - no gastric juice added. Withdrawn after 2 hr.

D - 2 ml. gastric juice added after 2 hr.

E - 10 ml. gastric juice added after 2 hr.

F - no gastric juice added. Withdrawn after 6 hr.

G - 2 ml. gastric juice added after 6 hr.

H - 10 ml. gastric juice added after 6 hr.

Except for C and F, all incubations are timed for 10 hr at 37° C.

Use I as standard.

Results

	% Uptake		Mean
Tubes A, A	90	90	90
B, B	3	4	4
C, C	58	60	59
D, D	59	62	61
E, E	54	60	57
F, F	85	85	85
G, G	86	85	86
H, H	84	84	84

Effect of dilution on the ability of 4 hr and 24 hr cultures of *E. coli* to take up vitamin B_{12} (Fig. 13).

Take tubes A, A; B, B each containing 1/15 ^{58}Co-vitamin B_{12} in 1 ml. water.

Take tubes C, C; D, D; E, E; F, F; G, G each containing 1/15 ug. ^{58}Co-vitamin B_{12} in 10 ml. water.

Take tubes H, H containing 1/15 ug. ^{58}Co-vitamin B_{12} in 20 ml. water.

Prepare 10 x 24 hr (overnight) 10 ml. cultures of *E. coli* as described previously.

To tubes A, C and H add 4 hr growing cultures.

To tubes B, D, E, F and G add 24 hr cultures.

Incubate at 37° C A, B, C, D and H for 1 hour
E for 2 hours
F for 3 hours
G for 4 hours

Centrifuge as before.

Compare radioactivity with appropriate standard

(i) 1/15 ug. ^{58}Co-vitamin B_{12} in 11 ml. water

(ii) " " " 20 ml. "

(iii) " " " 30 ml. "

Results

	% Uptake		Mean	
Tubes A, A	86	90	88	4 hr cultures
C, C	89	89	89	
H, H	90	88	89	
B, B	17	21	19	24 hr cultures
D, D	20	22	21	
E, E	22	26	24	
F, F	29	30	30	
G, G	34	34	34	

Effect of heat (56° C for 10 min.) on the ability of a 4 hr and a 24 hr culture of E. coli to take up vitamin B_{12} (Fig. 14).

(i) Take tubes A_1, A_2, A_3 containing 10 ml. broth.
Inoculate with 1 ml. overnight culture and grow for 4 hr at 37° C.

Take tubes B_1, B_2, B_3, B_4 containing 10 ml. broth.
Inoculate with 1 ml. overnight culture and grow for 3 hr 55 min. at 37° C.

At 3 hr 55 min. place tubes B in a waterbath at 56° C for 10 min.
At 4 hr add 1/15 ug. of ^{58}Co-vitamin B_{12} in 0.1 ml. water to tubes A.
At 4 hr 5 min. add 1/15 ug. of ^{58}Co-vitamin B_{12} in 0.1 ml. water to tubes B.

Incubate 1 hour at 37° C.
Centrifuge and measure uptake as before in tubes A_1 - A_3 and B_1 - B_3.
Standard - 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water added to 10 ml. broth + 1 ml. water.
Set up 1 ml. B_4 in broth to test viability of organisms.

(ii) Proceed as above but use 24 hr instead of 4 hr cultures.

Results

	4 hr Cultures % Uptake		24 hr Cultures % Uptake
Tubes A_1	86	Tubes A_1	9
A_2	85	A_2	11
A_3	85	A_3	16
B_1	7	B_1	11
B_2	13	B_2	7
B_3	11	B_3	7

Note organism was shown to be viable after heating at 56° C for 10 minutes.

Uptake of vitamin B_{12} by 5 and 20 ml. cultures of *E. coli* grown for 4, 7 and 24 hr in the presence and absence of H.G.J. (Fig. 15).

Take tubes A, A; B, B; C, C; D, D; E, E; F, F containing 5 ml. broth.

Take tubes G, G; H, H; I, I; J, J; K, K; L, L containing 20 ml. broth.

Inoculate with 1 ml. overnight culture.

After 4 hr of growth add tubes A and G to 1 ml. H.G.J. containing 1/15 ug. ^{58}Co-vitamin B_{12}.

After 4 hr of growth add tubes B and H to 1 ml. water containing 1/15 ug. ^{58}Co-vitamin B_{12}.

After 7 hr of growth add tubes C and I to 1 ml. H.G.J. containing 1/15 ug. ^{58}Co-vitamin B_{12}.

After 7 hr of growth add tubes D and J to 1 ml. water containing 1/15 ug. ^{58}Co-vitamin B_{12}.

After 24 hr of growth add tubes E and K to 1 ml. H.G.J. containing 1/15 ug. ^{58}Co-vitamin B_{12}.

After 24 hr of growth add tubes F and L to 1 ml. water containing 1/15 ug. ^{58}Co-vitamin B_{12}.

Incubate all tubes for a further hour at 37° C.

Centrifuge as before and measure uptake.

Standard - 1/15 ug. ^{58}Co-vitamin B_{12} in 1 ml. broth + 1 ml. water.

Results

	% Uptake		Mean	
Tubes A, A	74	74	74	
B, B	94	94	94	
C, C	53	50	52	
D, D	51	47	49	5 ml. cultures
E, E	10	7	9	
F, F	6	6	6	
G, G	80	80	80	
H, H	97	97	97	
I, I	69	74	72	
J, J	91	92	92	20 ml. cultures
K, K	24	25	25	
L, L	30	29	30	

Uptake by E. coli in the presence of increasing quantities of vitamin B_{12} (Fig. 16).

Details as for experiment illustrated in Fig. 8 (p. 117) except for parallel tubes using twice and three times the usual amount of ^{58}Co-vitamin B_{12}, i.e. 1/15 ug., 2/15 ug., 3/15 ug.

Results

Vit. B_{12} added ug.	Tubes		% Uptake		Mean
1/15	A, A	Culture + 1 ml. H_2O	93	93	93
2/15	B, B		95	92	94
3/15	C, C		94	90	92
1/15	D, D	Culture + 1 ml. H.G.J.	40	40	40
2/15	E, E		48	54	51
3/15	F, F		61	63	62
1/15	G, G	Culture + 2 ml. H.G.J.	4	0	2
2/15	H, H		40	30	35
3/15	I, I		37	36	37
1/15	J, J	Culture + 6 ml. H.G.J.	1	0	1
2/15	K, K		3	3	3
3/15	L, L		1	1	1

Effect of added nutrition on the ability of *E. coli* to take up vitamin B_{12} in the presence of 1 ml. of H.G.J. (Fig. 17).

Take duplicate tubes as before, A, A; B, B; C, C; D, D; E, E; each containing 1 ml. H.G.J. and 1/15 ug. ^{58}Co-vitamin B_{12}.

Add a 4 hr growing culture to each tube and incubate at 37° C.

At the end of 1 hour:

Centrifuge as usual and measure uptake in A.

Add 6 ml. vitamin B_{12} free medium to tubes B and C.

Incubate at 37° C.

At end of further 4 hours:

Centrifuge and measure uptake in B and D.

Add 6 ml. vitamin B_{12} free medium to tubes C.

At end of further 5 hours:

Centrifuge and measure uptake in C and E.

Results

	% Uptake		Mean	
Tubes A, A	16	17	17	Original culture at 1 hr.
B, B	25	24	25	Original culture + 6 ml. medium at 5 hr.
C, C	39	41	40	Original culture + 12 ml. medium at 10 hr.
D, D	19	18	19	Original culture at 5 hr.
E, E	22	18	20	Original culture at 10 hr.

Note Standards for A, D and E - 1/15 ug. ^{58}Co-vitamin B_{12} in 10 ml. broth + 1 ml. water.

Standards for B - 1/15 ug. ^{58}Co-vitamin B_{12} in 10 ml. broth + 7 ml. water.

Standards for C - 1/15 ug. ^{58}Co-vitamin B_{12} in 10 ml. broth + 13 ml. water.

Effect of dilution and added nutrition on the ability of *E. coli* to take up vitamin B_{12} in the presence of 1 ml. of H.G.J. (Fig. 18).

Experiment similar to that just described except that extra tubes were added for the addition of water instead of vitamin B_{12} free medium, and for the optical density measurements.

Results

	% Uptake		Mean	
Tubes A, A	12	11	12	Original culture at 1 hr.
B, B	18	17	18	Original culture + 6 ml. medium at 5 hr.
C, C	18	12	15	Original culture + 6 ml. water at 5 hr.
D, D	31	29	30	Original culture + 12 ml. medium at 10 hr.
E, E	17	15	16	Original culture + 12 ml. water at 10 hr.
F, F	15	12	14	Original culture at 5 hr.
G, G	14	12	13	Original culture at 10 hr.

Effect of adding growing culture of E. coli at various pH values to vitamin B_{12} in 1 ml. H.G.J. and 1 ml. water (Fig. 19).

Set up duplicate tubes A - I containing 1 ml. water.

Set up duplicate tubes J - R containing 1 ml. H.G.J.

Adjust pH of A to 2; B to 3; C to 4; D to 5; E to 6; F to 7; G to 8; H to 9; I to 10.

Adjust pH of J to 2; K to 3; L to 4; M to 5; N to 6; O to 7; P to 8; Q to 9; R to 10.

(pH values were in fact adjusted using concentrated acid on alkali in 5 ml. aliquots. The 1 ml. aliquots were measured out subsequently).

To each add 1/15 ug. ^{58}Co-vitamin B_{12} and incubate to 37° C.

To each add 4 hr 10 ml. culture after bringing the pH of the culture to that of the sample to which it is to be added.

Incubate further hour and measure uptake as before.

Standard - 1/15 ug. ^{58}Co-vitamin B_{12} in 10 ml. broth + 1 ml. water.

Results

	% Uptake		Mean
Tubes A, A	2	2	2
B, B	0	2	1
C, C	0	4	2
D, D	86	86	86
E, E	89	91	90
F, F	91	91	91
G, G	91	90	91
H, H	90	87	89
I, I	10	8	9
J, J	0	0	0
K, K	0	0	0
L, L	0	0	0
M, M	14	11	13
N, N	18	21	20
O, O	23	24	24
P, P	25	27	26
Q, Q	25	25	25
R, R	4	4	4

Experiments to measure the effect of enzymes on the binding activity of H.G.J. for E. coli before and after the addition of vitamin B_{12} (Figs 20 - 28).

In these experiments the effect of the enzymes was measured on duplicate aliquots of 1, 2 and 6 ml. of gastric juice.

The principle of the experiments is as set out in Tables 23 and 24. The effect of Pepsin on the binding activity of gastric juice before and after vitamin B_{12} had been added to the juice was studied as illustrated in Table 23. The effect of Pepsin followed by Trypsin on the binding activity of gastric juice before and after vitamin B_{12} was added to the juice was studied as illustrated in Table 24. Other enzymes can be substituted for these providing suitable pH adjustments are made. When Trypsin and Chymotrypsin were used in a single experiment they were used simultaneously. Because of the pH adjustments which were necessary, the ^{58}Co-vitamin B_{12} in these experiments was added to the gastric juice at either pH2 or pH7. As can be seen from Fig. 31 the pH at which the addition was made to the juice did not affect the subsequent uptake by a microorganism. Note that the method used to measure intrinsic factor permits determinations only on juice to which isotope has not been previously added.

TABLE 23

Effect of Pepsin on Binding Activity of H.G.J. before and after the addition of Vitamin B_{12} to the Juice

Take volume of H.G.J. which has been alkali inactivated (i.e. is at pH7) and bring to pH2. Dispense as follows:-

1.	1 ml H.G.J.	Add 2.5 mgs pepsin in 0.1 ml water; Incubate 4 hrs. 37°C; Add 1/15 ugm^{58} Co-B_{12} in 0.1 ml water; Alkali inactivate the pepsin.
2.	1 ml H.G.J.	
3.	1 ml H.G.J.	
4.	2 ml H.G.J.	Add 5 mgs pepsin in 0.2 ml water; " " " " " " " " "
5.	2 ml H.G.J.	
6.	2 ml H.G.J.	
7.	6 ml H.G.J.	Add 15 mgs pepsin in 0.6 ml water; " " " " " " " " "
8.	6 ml H.G.J.	
9.	6 ml H.G.J.	
10.	1 ml H.G.J.	Add 0.1 ml water; Incubate 4 hrs. 37°C; Add 1/15 ugm^{58} Co-Vit.B_{12} in 0.1 ml water; Alkali inactivate as if pepsin was present.
11.	1 ml H.G.J.	
12.	1 ml H.G.J.	
13.	2 ml H.G.J.	Add 0.2 ml water; " " " " " " " " " "
14.	2 ml H.G.J.	
15.	2 ml H.G.J.	
16.	6 ml H.G.J.	Add 0.6 ml water; " " " " " " " " " "
17.	6 ml H.G.J.	
18.	6 ml H.G.J.	
19.	1 ml H.G.J.	Add 1/15 ugm^{58} Co-B_{12} in 0.1 ml water; Add 2.5 mgs pepsin in 0.1 ml water; Incubate 4 hrs. at 37°C; Alkali inactivate pepsin.
20.	1 ml H.G.J.	
21.	2 ml H.G.J.	" " " " " Add 5 mgs pepsin in 0.2 ml water; " " " " " "
22.	2 ml H.G.J.	
23.	6 ml H.G.J.	" " " " " Add 15 mgs pepsin in 0.6 ml water; " " " " " "
24.	6 ml H.G.J.	
25.	1 ml H.G.J.	" " " " " Add 0.1 ml water; " " " Alkali inactivate as if pepsin was present.
26.	1 ml H.G.J.	
27.	2 ml H.G.J.	" " " " " Add 0.2 ml water; " " " " " "
28.	2 ml H.G.J.	
29.	6 ml H.G.J.	" " " " " Add 0.6 ml water; " " " " " "
30.	6 ml H.G.J.	

Withdraw tubes 3, 6, 9, 12, 15 and 18 for instrinsic factor determination.

To other tubes add a 10 ml 4 hr. culture.

Incubate 1 hr. at 37°C.

Measure uptake using standards of suitable volume containing 1/15 ugm^{58} Co-Vit. B_{12}

TABLE 24

Effect of Pepsin followed by Trypsin on the Binding Activity of H.G.J. before and after the addition of Vit.B_{12} to the Juice.

Take volume of H.G.J. which has been alkali inactivated (i.e. is at pH7) and bring to pH2. Dispense as follows:

1.	1 ml H.G.J.	Add Pepsin as described in Table 23 and incubate 4 hrs. at 37°C.	Alkali inactivate Pepsin but bring pH back to 8 instead of 7	Add 2.5 mgs Trypsin in 0.1 ml water; Incubate 4 hrs. at 37°C.; Adjust pH to 7 and add 1/15 ugm^{58} Co-B_{12} in 0.1 ml water.
2.	1 ml H.G.J.			Add 5 mgs Trypsin in 0.2 ml water; " " " " " " " "
3.	1 ml H.G.J.			Add 15 mgs Trypsin in 0.6 ml water; " " " " " " " "
4.	2 ml H.G.J.			
5.	2 ml H.G.J.			
6.	2 ml H.G.J.			
7.	6 ml H.G.J.			
8.	6 ml H.G.J.			
9.	6 ml H.G.J.			
10.	1 ml H.G.J.	Add water as described in Table 23 and incubate 4 hrs. at 37°C.	Alkali inactivate as if Pepsin was present but bring pH back to 8 instead of 7	Add 0.1 ml water; " " " " " " " "
11.	1 ml H.G.J.			
12.	1 ml H.G.J.			Add 0.2 ml water; " " " " " " " "
13.	2 ml H.G.J.			
14.	2 ml H.G.J.			Add 0.6 ml water; " " " " " " " "
15.	2 ml H.G.J.			
16.	6 ml H.G.J.			
17.	6 ml H.G.J.			
18.	6 ml H.G.J.			
19.	1 ml H.G.J.	Add 1/15 ugm^{58}Co-B_{12} as described in Table 23	Add Pepsin as described in Table 23 and incubate 4 hrs. at 37°C.	Alkali inactivate Pepsin but bring pH back to 8 instead of 7
20.	1 ml H.G.J.			Add 2.5 mgs Trypsin in 0.1 ml water; Incubate 4 hrs. at 37°C.; Adjust pH to 7.
21.	2 ml H.G.J.			
22.	2 ml H.G.J.			Add 5 mgs Trypsin in 0.2 ml water; " " " " "
23.	6 ml H.G.J.			
24.	6 ml H.G.J.			Add 15 mgs Trypsin in 0.6 ml water; " " " " "
25.	1 ml H.G.J.		Add water as described in Table 23 and incubate 4 hrs. at 37°C.	Alkali inactivate as if Pepsin was present but bring pH back to 8 instead of 7
26.	1 ml H.G.J.			Add 0.1 ml water; " " " " "
27.	2 ml H.G.J.			
28.	2 ml H.G.J.			Add 0.2 ml water; " " " " "
29.	6 ml H.G.J.			
30.	6 ml H.G.J.			Add 0.6 ml water; " " " " "

Withdraw tubes 3, 6, 9, 12, 15 and 18 for intrinsic factor determination.

To other tubes add a 10 ml 4 hr. growing culture.

Incubate 1 hr. at 37°C.

Measure uptake using standards of suitable volume containing 1/15 ugm^{58} Co-Vit.B_{12}

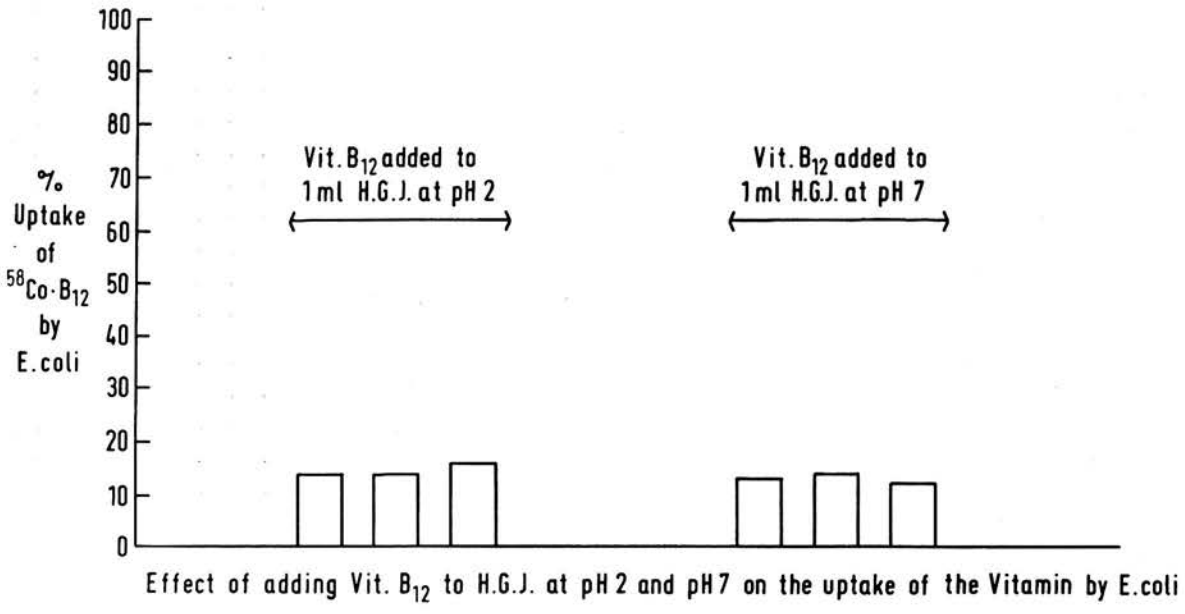


Figure 31. Effect of adding vitamin B_{12} to human gastric juice at pH2 and pH7 on the uptake of vitamin B_{12} by E. coli.

Effect of Pepsin on binding activity of H.C.J. (Fig. 20).

Results

	% Uptake		Mean
Control - no H.C.J. added	93	94	94
H.C.J. 1 ml./Pepsin 4 hr/B_{12}	54	50	52
H.C.J. 2 ml./Pepsin 4 hr/B_{12}	18	14	16
H.C.J. 6 ml./Pepsin 4 hr/B_{12}	6	2	4
H.C.J. 1 ml./H_2O 4 hr/B_{12}	3	2	3
H.C.J. 2 ml./H_2O 4 hr/B_{12}	7	1	4
H.C.J. 6 ml./H_2O 4 hr/B_{12}	4	4	4
H.C.J. 1 ml./B_{12}/Pepsin 4 hr	9	14	12
H.C.J. 2 ml./B_{12}/Pepsin 4 hr	2	9	6
H.C.J. 6 ml./B_{12}/Pepsin 4 hr	5	4	5
H.C.J. 1 ml./B_{12}/H_2O 4 hr	0	3	2
H.C.J. 2 ml./B_{12}/H_2O 4 hr	0	0	0
H.C.J. 6 ml./B_{12}/H_2O 4 hr	1	3	2

Intrinsic factor determinations prior to peptic digestion - 76.0 ngm./ml.

Intrinsic factor determinations after peptic digestion - 18.7) 14.7) 17.8) 16.9 ngm./ml.

Intrinsic factor determinations in controls - 57.2) 48.2) 64.8) 56.8 ngm./ml.

Effect of Trypsin on binding activity of H.G.J. (Fig. 21).

Effect of Chymotrypsin on binding activity of H.G.J. (Fig. 22).

Results

	% Uptake		Mean
Control - no H.G.J. added	90	89	90
H.G.J. 1 ml./Trypsin 4 hr/B_{12}	4	0	2
H.G.J. 1 ml./Chymotrypsin 4 hr/B_{12}	14	9	12
H.G.J. 2 ml./Trypsin 4 hr/B_{12}	0	0	0
H.G.J. 2 ml./Chymotrypsin 4 hr/B_{12}	0	0	0
H.G.J. 6 ml./Trypsin 4 hr/B_{12}	0	0	0
H.G.J. 6 ml./Chymotrypsin 4 hr/B_{12}	0	0	0
H.G.J. 1 ml./H_2O 4 hr/B_{12}	0	0	0
H.G.J. 2 ml./H_2O 4 hr/B_{12}	0	0	0
H.G.J. 6 ml./H_2O 4 hr/B_{12}	0	0	0
H.G.J. 1 ml./B_{12}/Trypsin 4 hr	1	0	1
H.G.J. 1 ml./B_{12}/Chymotrypsin 4 hr	4	1	3
H.G.J. 2 ml./B_{12}/Trypsin 4 hr	0	0	0
H.G.J. 2 ml./B_{12}/Chymotrypsin 4 hr	0	0	0
H.G.J. 6 ml./B_{12}/Trypsin 4 hr	0	0	0
H.G.J. 6 ml./B_{12}/Chymotrypsin 4 hr	0	0	0
H.G.J. 1 ml./B_{12}/H_2O 4 hr	0	0	0
H.G.J. 2 ml./B_{12}/H_2O 4 hr	0	0	0
H.G.J. 6 ml./B_{12}/H_2O 4 hr	0	0	0

Intrinsic factor determination prior to digestion - 76.0 ngm./ml.

Intrinsic factor determination after digestion with Trypsin -

64.6)
58.8) 61.1 ngm./ml.
59.6)

Intrinsic factor determination after digestion with Chymotrypsin -

52.8)
52.9) 53.5 ngm./ml.
54.8)

Intrinsic factor determination in controls - 58.5)
60.3) 58.7 ngm./ml.
57.2)

Effect of Trypsin and Chymotrypsin on binding activity of H.G.J. (Fig. 23).

Results

	% Uptake		Mean
Control - no H.G.J. added	90	88	89
H.G.J. 1 ml./Trypsin Chymotrypsin 4 hr/B_{12}	16	14	15
H.G.J. 2 ml./Trypsin Chymotrypsin 4 hr/B_{12}	2	2	2
H.G.J. 6 ml./Trypsin Chymotrypsin 4 hr/B_{12}	4	4	4
H.G.J. 1 ml./H_2O 4 hr/B_{12}	0	4	2
H.G.J. 2 ml./H_2O 4 hr/B_{12}	0	0	0
H.G.J. 6 ml./H_2O 4 hr/B_{12}	3	5	4
H.G.J. 1 ml./B_{12}/Trypsin Chymotrypsin 4 hr	1	2	2
H.G.J. 2 ml./B_{12}/Trypsin Chymotrypsin 4 hr	0	2	1
H.G.J. 6 ml./B_{12}/Trypsin Chymotrypsin 4 hr	8	2	5
H.G.J. 1 ml./B_{12}/H_2O 4 hr	2	2	2
H.G.J. 2 ml./B_{12}/H_2O 4 hr	0	0	0
H.G.J. 6 ml./B_{12}/H_2O 4 hr	6	8	7

Intrinsic factor determination prior to digestion - 76.0 ngm./ml.

Intrinsic factor determination after digestion with Trypsin and Chymotrypsin - 47.4) 43.7 ngm./ml.
39.3)

Intrinsic factor determination in controls - 60.4)
58.0) 56.2 ngm./ml.
53.2)

Effect of Pepsin followed by Trypsin on binding activity of H.G.J. (Fig. 24).

Effect of Pepsin followed by Chymotrypsin on binding activity of H.G.J. (Fig. 25).

Effect of Pepsin followed by Trypsin and Chymotrypsin on binding activity of H.G.J. (Fig. 26).

Results

	% Uptake		Mean
Control - no H.G.J. added	92	96	94
H.G.J. 1 ml./Pepsin 4 hr/Trypsin 4 hr/B_{12}	66	61	64
H.G.J. 2 ml./Pepsin 4 hr/Trypsin 4 hr/B_{12}	35	33	34
H.G.J. 6 ml./Pepsin 4 hr/Trypsin 4 hr/B_{12}	14	10	12
H.G.J. 1 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	6	6	6
H.G.J. 2 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	1	1	1
H.G.J. 2 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	6	3	5
H.G.J. 1 ml./B_{12}/Pepsin 4 hr/Trypsin 4 hr	13	-	13
H.G.J. 2 ml./B_{12}/Pepsin 4 hr/Trypsin 4 hr	4	0	2
H.G.J. 6 ml./B_{12}/Pepsin 4 hr/Trypsin 4 hr	5	2	4
H.G.J. 1 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	0	2	1
H.G.J. 2 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	0	0	0
H.G.J. 6 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	8	1	5
H.G.J. 1 ml./Pepsin 4 hr/Chymotrypsin 4 hr/B_{12}	69	-	69
H.G.J. 2 ml./Pepsin 4 hr/Chymotrypsin 4 hr/B_{12}	50	50	50
H.G.J. 6 ml./Pepsin 4 hr/Chymotrypsin 4 hr/B_{12}	17	18	18
H.G.J. 1 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	16	11	14
H.G.J. 2 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	7	7	7
H.G.J. 6 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	10	9	10

	% Uptake		Mean
H.G.J. 1 ml./B_{12}/Pepsin 4 hr/Chymotrypsin 4 hr	15	25	20
H.G.J. 2 ml./B_{12}/Pepsin 4 hr/Chymotrypsin 4 hr	6	7	7
H.G.J. 6 ml./B_{12}/Pepsin 4 hr/Chymotrypsin 4 hr	8	4	6
H.G.J. 1 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	6	0	3
H.G.J. 2 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	0	0	0
H.G.J. 6 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	6	4	5
H.G.J. 1 ml./Pepsin 4 hr/Trypsin Chymotrypsin 4 hr/B_{12}	73	73	73
H.G.J. 2 ml./Pepsin 4 hr/ " "	68	69	69
H.G.J. 6 ml./Pepsin 4 hr/ " "	24	26	25
H.G.J. 1 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	13	11	12
H.G.J. 2 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	4	3	4
H.G.J. 6 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	8	3	6
H.G.J. 1 ml./B_{12}/Pepsin 4 hr/Trypsin Chymo-trypsin 4 hr	26	22	24
H.G.J. 2 ml./B_{12}/Pepsin 4 hr/ " "	7	5	6
H.G.J. 6 ml./B_{12}/Pepsin 4 hr/ " "	2	3	3
H.G.J. 1 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	2	2	2
H.G.J. 2 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	0	0	0
H.G.J. 6 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	6	2	4

Intrinsic factor determination prior to digestion - 76.0 ngm./ml.

Intrinsic factor determination after digestion with Pepsin and Trypsin - 14.0)
13.3) 16.1 ngm./ml.
20.9)

Intrinsic factor determination in Controls - 47.8)
49.4) 50.5 ngm./ml.
54.3)

Intrinsic factor determination after digestion with Pepsin and Chymotrypsin - 17.6, 16.9) 17.0 ngm./ml.

Intrinsic factor determination in Controls - 50.5, 57.4) 53.9 ngm./ml.

Intrinsic factor determination after digestion with Pepsin and Trypsin and Chymotrypsin - 17.1, 14.2, 16.1) 15.8 ngm./ml.

Intrinsic factor determination in Controls - 56.9, 48.9, 52.8) 50.8 ngm./ml.

Effect of Pepsin followed by Trypsin and Chymotrypsin on binding activity of H.G.J. (Fig. 27).

Results

	% Uptake		Mean
Control - no H.G.J. added	90	86	88
H.G.J. 1 ml./Pepsin 4 hr/Trypsin Chymotrypsin 4 hr/B_{12}	90	91	91
H.G.J. 2 ml./Pepsin 4 hr/ " "	87	86	87
H.G.J. 6 ml./Pepsin 4 hr/ " "	73	73	73
H.G.J. 1 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	29	28	29
H.G.J. 2 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	14	12	13
H.G.J. 6 ml./H_2O 4 hr/H_2O 4 hr/B_{12}	6	10	8
H.G.J. 1 ml./B_{12}/Pepsin 4 hr/Trypsin Chymotrypsin 4 hr	35	37	36
H.G.J. 2 ml./B_{12}/Pepsin 4 hr/ " "	31	30	31
H.G.J. 6 ml./B_{12}/Pepsin 4 hr/ " "	27	32	30
H.G.J. 1 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	2	0	1
H.G.J. 2 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	2	6	4
H.G.J. 6 ml./B_{12}/H_2O 4 hr/H_2O 4 hr	9	9	9

Intrinsic factor determination prior to digestion - 27.0 ngm./ml.

Intrinsic factor determination after digestion - 0.0, 5.4) 2.7 ngm./ml.

Intrinsic factor determination in Controls - 23.1, 19.5) 21.3 ngm./ml.

Effect of Pepsin on binding activity of H.G.J. from patient with jejunal diverticulosis (Fig. 28).

Results

	% Uptake		Mean
Control - no H.G.J. added	72	73	73
H.G.J. 1 ml./Pepsin 4 hr/B_{12}	78	74	76
H.G.J. 2 ml./Pepsin 4 hr/B_{12}	71	69	70
H.G.J. 6 ml./Pepsin 4 hr/B_{12}	38	-	38
H.G.J. 1 ml./H_2O 4 hr/B_{12}	39	32	36
H.G.J. 2 ml./H_2O 4 hr/B_{12}	5	2	4
H.G.J. 6 ml./H_2O 4 hr/B_{12}	3	-	3
H.G.J. 1 ml./B_{12}/Pepsin 4 hr	34	36	35
H.G.J. 2 ml./B_{12}/Pepsin 4 hr	29	25	27
H.G.J. 6 ml./B_{12}/Pepsin 4 hr	10	-	10
H.G.J. 1 ml./B_{12}/H_2O 4 hr	22	23	23
H.G.J. 2 ml./B_{12}/H_2O 4 hr	6	0	6
H.G.J. 6 ml./B_{12}/H_2O 4 hr	0	-	0

Intrinsic factor determination before digestion - 54.4 ngm./ml.

Intrinsic factor determination after digestion - 1.4) 2.3) 3.2) 2.3 ngm./ml.

Intrinsic factor determination in Controls - 28.3) 26.6) 27.5 ngm./ml.

Effect of autodigestion for 4 and 6 hr on the binding activity of H.G.J. (Figs 29 and 30).

Arrange experiment for a morning when acid secretion test under maximal intestinal stimulation is being carried out on a suitable subject. The gastric juice is collected under ice as usual and the experiment carried out as soon as the juice is available after passing it through several layers of gauze.

Remove aliquot, alkali inactivate Pepsin and store -20° C for intrinsic factor determination.

Adjust the pH of the rest of the juice to 2.0.

Dispense the following aliquots:

A_o = 1 ml.	E_o = 2 ml.	I_o = 6 ml.	Provide duplicate samples of B, D, F, H, J, L for intrinsic factor determination after appropriate period of incubation.
A_o = 1 ml.	E_o = 2 ml.	I_o = 6 ml.	
A = 1 ml.	E = 2 ml.	I = 6 ml.	
B = 1 ml.	F = 2 ml.	J = 6 ml.	
C = 1 ml.	G = 2 ml.	K = 6 ml.	
D = 1 ml.	H = 2 ml.	L = 6 ml.	

Proceed as follows:

(1) Add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water to A_o, E_o, I_o, A, B, E, F, I. J.

(2) Alkali inactivate A_o, E_o, I_o immediately and store at -20° C.

(3) Incubate A, C, E, G, I, K for 4 hours at 37° C.

Add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water to C, G, K.

Alkali inactivate all samples and store at -20° C.

(4) Incubate B, D, F, H, J, L for 6 hours at 37° C.

Add 1/15 ug. ^{58}Co-vitamin B_{12} in 0.1 ml. water to D, H, L.

Alkali inactivate all samples and store at -20° C.

Measure uptake as described previously using a 1 ml. overnight culture.

Incubate 10 hours.

For results see Table 25.

The results of an identical experiment using juice of lower initial intrinsic factor concentration are given in Table 26.

TABLE 25

Results of expirement showing effect of autodigestion for 4 and 6 hrs. on the binding activity of H.G.J. (Fig. 29)

Control Tubes - No H.G.J. added 94% 94%

Mls H.G.J. added	Juice not incubated	Juice incubated 4 hrs.		Juice incubated 6 hrs.	
	1/15 ugm ^{58}Co-B_{12} added prior to inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added after inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added prior to inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added after inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added prior to inactivation of pepsin
	% uptake	% uptake	% uptake	% uptake	% uptake
1 ml.	11	65	17	80	34
2 ml.	6	37	15	66	25
6 ml.	1	12	5	17	6

Intrinsic factor determination in unincubated juice 67.8 ngm. per ml.
Intrinsic factor determination in juice autodigested for 4 hrs. 17.9 ngm. per ml.
Intrinsic factor determination in inactivated control after 4 hrs. 62.3 ngm. per ml.
Intrinsic factor determination in juice autodigested for 6 hrs. 11.4 ngm. per ml.
Intrinsic factor determination in inactivated control juice after 6 hrs. 62.5 ngm. per ml.

TABLE 26

Results of expirement showing effect of autodigestion for 4 and 6 hrs. on the binding activity of H.G.J. (Fig. 30)

Control Tubes - No H.G.J. added 93% 92%

Mls H.G.J. added	Juice not incubated	Juice incubated 4 hrs.		Juice incubated 6 hrs.	
	1/15 ugm ^{58}Co-B_{12} added prior to inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added after inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added prior to inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added after inactivation of pepsin	1/15 ugm ^{58}Co-B_{12} added prior to inactivation of pepsin
	% uptake	% uptake	% uptake	% uptake	% uptake
1 ml.	32	83	36	88	53
2 ml.	11	72	20	84	31
6 ml.	4	37	15	67	21

Intrinsic factor determination in unincubated juice - 46.7 ngm. per ml.
Intrinsic factor determination in juice autodigested for 4 hrs. - 0 ngm. per ml.
Intrinsic factor determination in inactivated control after 4 hrs. - 43.0 ngm. per ml.
Intrinsic factor determination in juice autodigested for 6 hrs. - 0 ngm. per ml.
Intrinsic factor determination in inactivated control after 6 hrs. - 44.5 ngm. per ml.

APPENDIX II

The Effect of Berkefeld Filtration on the Binding Activity of Human Gastric Juice

The Effect of Berkefeld Filtration on the Binding Activity of Human Gastric Juice

It is known that filtration of human gastric juice through a Seitz filter causes it to lose intrinsic factor activity while Berkefeld filtration is said not to do this (Taylor, Castle, Heinle and Adams, 1938; Hall, Morgan and Campbell, 1949; Ungley, 1950) but there is little recent information on this subject (Glass, 1963). During the present work involving the study of the uptake of micro-organisms in the presence of gastric juice, the problem of sterilising the juice was encountered. This prompted further study of the effect of Berkefeld filtration on the binding activity of gastric juice, particularly after it was noted that after filtration the binding activity of gastric juice tended to be somewhat variable.

The methods are as have already been described. For the filtration work six new standard filter candles (British Berkefeld Filters Ltd., No. 8) were used. They were prepared and cleaned for the experiments as instructed by the makers by boiling in water for 20 minutes. They were then autoclaved at 15 lb. pressure for 10 minutes.

In Table 27 are illustrated the results of bacterial uptake studies in the presence of increasing volumes of gastric juice which had been filtered at pH7 as compared to the uptakes in the presence of unfiltered juice. The uptakes in the presence of filtered juice are slightly higher and though the differences are small they are reproducible. From Table 28 it will be seen that the binding activity of gastric juice was more susceptible to peptic digestion after it had been filtered. In Table 29 it will be seen from the uptake studies that the loss of binding activity becomes more pronounced as the pH at which the juice is filtered is dropped.

Figs 32 and 33 show the results of two similar experiments in which intrinsic factor determinations were made on the gastric juice before and after filtration. Sixty ml. of juice were passed through each filter. The intrinsic factor concentration of the juice prior to filtration was 65.6 ngm./ml. Figs 34 and 35 illustrate the results of similar experiments, filtration being carried out at different pH values. A different pool of gastric juice (intrinsic factor concentration 49.7 ngm./ml.) was employed on this occasion. These figures on the whole confirm the observations made in the vitamin B_{12} uptake experiments and indicate that the loss of binding activity noted there was due to loss of intrinsic factor activity.

The findings confirm the claim of previous workers in that

TABLE 27

Volume of gastric juice added (ml.)	Percentage uptake of radioactivity	
	G.J. filtered at pH7	Unfiltered G.J.
0	93.0	93.0
2	18.0	12.0
6	11.0	10.0
10	10.0	7.0

The percentage of uptake of labelled cyanocobalamin by E. coli in the presence of increasing volumes of unfiltered gastric juice and of juice filtered at pH7

TABLE 28

Volume of gastric juice added (ml.)	Percentage uptake of radioactivity	
	G.J. exposed to peptic digestion for 4 hours prior to filtration at pH7	G.J. exposed to peptic digestion for 4 hours after filtration at pH7
0	84.0	84.0
2	33.5	53.9
6	4.5	17.5
10	3.1	8.3

The percentage uptake of labelled cyanocobalamin by *E. coli* in the presence of gastric juice which has been exposed to peptic digestion before and after filtration

TABLE 29

Volume of gastric juice added (ml.)	Percentage uptake of radioactivity G.J. filtered at various pH values					
	pH2	pH4	pH6	pH7	pH8	pH10
0	90.9	90.9	90.9	90.9	90.9	90.9
2	89.0	80.8	20.0	16.0	12.8	13.5
6	84.0	64.3	8.0	7.5	7.8	5.2
10	81.0	54.9	6.0	2.4	6.4	3.1

The percentage uptake of labelled cyanocobalamin by E. coli in the presence of increasing volumes of gastric juice filtered at different pH values

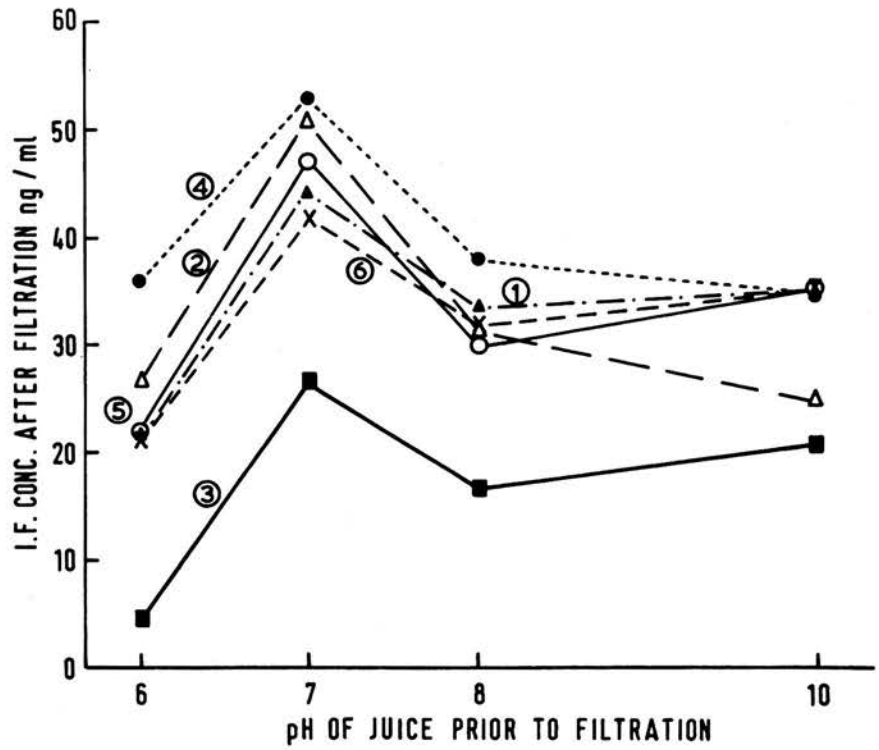


Figure 32. Effect of Berkefeld filtration of human gastric juice at pH values 6 - 10 on the intrinsic factor concentration of the juice. The intrinsic factor concentration of the juice prior to filtration was 65.6 ngm. per ml.

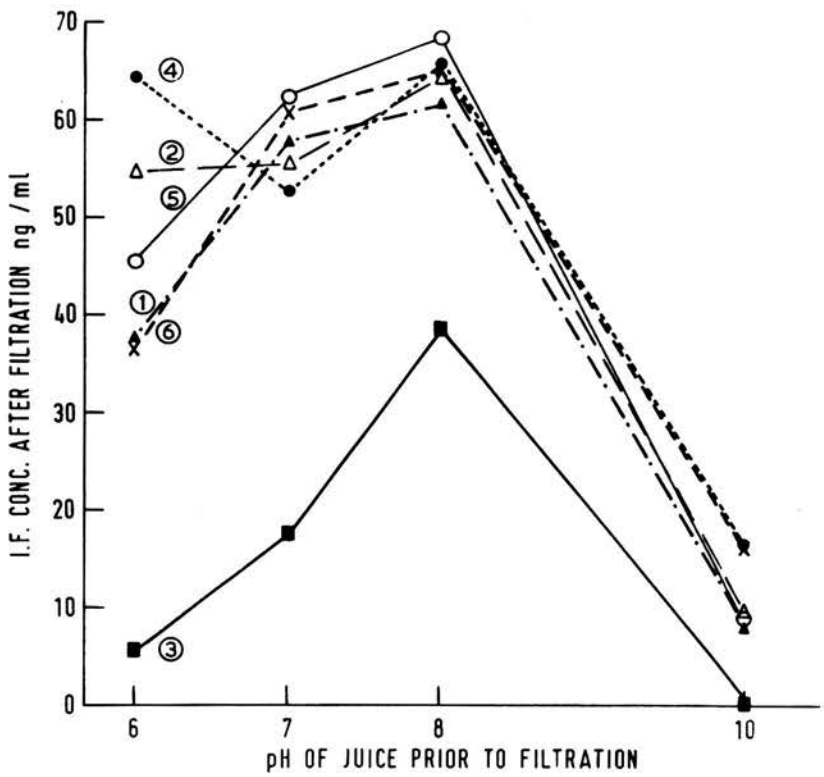


Figure 33. Effect of Berkefeld filtration of human gastric juice at pH values 6 - 10 on the intrinsic factor concentration of the juice. The intrinsic factor concentration of the juice prior to filtration was 65.6 ngm. per ml.

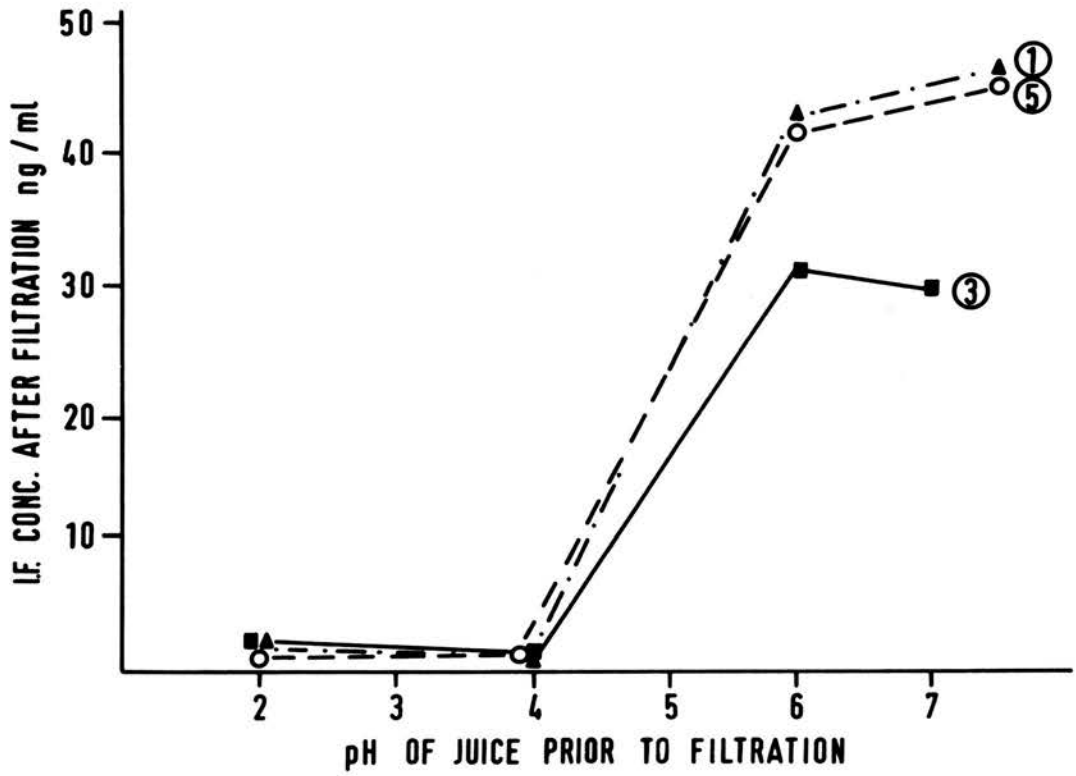


Figure 34. Effect of Berkefeld filtration of human gastric juice at pH values 2 - 7 on the intrinsic factor concentration of the juice. The intrinsic factor concentration of the juice prior to filtration was 49.7 ngm. per ml.

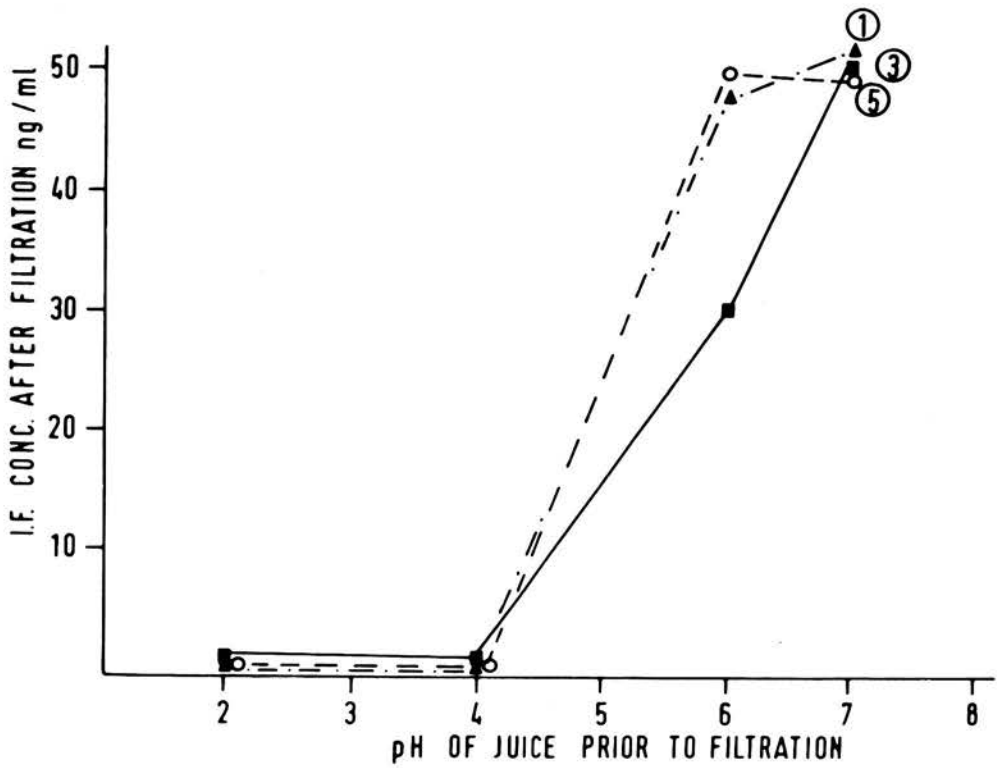


Figure 35. Effect of Berkefeld filtration of human gastric juice at pH values 2 - 7 on the intrinsic factor concentration of the juice. The intrinsic factor concentration of the juice prior to filtration was 49.7 ngm. per ml.

gastric juice which has been filtered through a Berkefeld filter still contains intrinsic factor. This is true, however, only in the pH range 7 - 8. At pH values above and below this range there tends to be some loss of intrinsic factor activity and this is complete in the lower pH range. It should be recalled here that intrinsic factor is known to be stable up to a pH of 11.5 (Glass, 1963). There appears to be considerable variability not only between one filter and another but also between the results obtained by the same filter used on a different occasion. Some filters, such as No. 3 in the present work, may remove considerable quantities of intrinsic factor even in the more favourable pH range.

The manner by which the filters bring about their effect is puzzling but an important clue may be contained in the observations made on the pH of the filtrate. This tends towards neutrality regardless of the pH of the juice prior to filtration. Thus when juice is filtered at pH2, the pH of the effluent has changed to between 7 and 8. The application of negative presence to the filters has not significantly affected the results obtained and it has not been possible to wash the intrinsic factor presumably left behind, out of the filters through which juice has been passed at low pH with either alkali or water. Prolonging the period of filtration does not result in any significant change in the intrinsic factor concentrations of the filtered juice though the intrinsic factor content of initial aliquots of juice even when filtered at pH7 may be very low.

Gastric juice is sometimes used as a source of intrinsic factor

in tests of vitamin B_{12} absorption and Berkefeld filtration is a popular method of preparing it for this procedure (Taylor et al., 1938; Hall et al., 1949; Ungley, 1950). The present work suggests that occasionally this could lead to erroneous results. Thus, 50 ml. of gastric juice of intrinsic factor content 65.6 ngm./ml. if passed through the worst of our filters at pH7 would still contain 890 ngm. of intrinsic factor. This would usually be enough to correct impaired absorption of vitamin B_{12} in a patient with pernicious anaemia though more might be required in a patient with deficiency of intrinsic factor after gastric surgery (Ardeman and Chanarin, 1965). Error would of course be more likely if juice of a lower intrinsic factor concentration was being filtered. The effect of Berkefeld filtration in gastric juice in more subtle work certainly cannot be disregarded and consequently its use in the work described had to be abandoned.

Summary

The intrinsic factor content of berkefeld-filtered human gastric juice has been studied. This appears to vary with the pH at which filtration is carried out and also between individual filters. Significant losses of intrinsic factor may result from filtration and complete loss occurs when filtration is carried out at low pH. The most suitable pH for filtration appears to be in the range pH 7 - 8.

APPENDIX III

Published Papers

Published Papers

Bacterial changes in the small intestine in malabsorptive states and in pernicious anaemia. Dellipiani, A. W. and Girdwood, R. H. (1964). Clin. Sci., 26, 360.

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The significance of abnormal bacterial proliferation in the gastrointestinal tract after gastric surgery. Dellipiani, A. W. and Girdwood, R. H. (1967). Scand. J. Gastroent., 2, 161.

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BACTERIAL CHANGES IN THE SMALL INTESTINE IN MALABSORPTIVE STATES AND IN PERNICIOUS ANÆMIA

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THE extent of the colonization by bacteria of the small intestine of man in health and disease has for many years been a source of speculation, and several investigations have been carried out in which sampling tubes have been passed by mouth. The studies by Cregan and Hayward (1953), in which they sampled the small bowel contents directly at operation, have helped to clarify the position in normal subjects. In spite of this, as French (1961) points out in a recent review of the literature, no clear picture has emerged concerning the bacteriological status of the small intestine in disease.

We have been interested for some time in the possible relation of bacteria to small intestinal disease (Girdwood, 1950; 1955 *a*; 1955 *b*; 1959; Doig and Girdwood, 1960), and this paper reports the findings of the bacterial flora in four different groups of patients who were studied by an intubation technique.

In an attempt to elucidate what part fæcal type organisms isolated from these patients might be playing in the absorption of vitamin B_{12}, the uptake of labelled cyanocobalamin by these organisms was studied *in vitro*.

MATERIAL AND METHODS

The patients studied were eight with malabsorptive disease, fifteen with pernicious anæmia and twenty-eight with surgical or congenital blind or stagnant loops of the small intestine.

Diagnosis of malabsorptive disease was made on the basis of examination of the stool for fat and nitrogen, the xylose absorption test, the folic acid absorption test of Girdwood (1960), radiological examination of the small intestine and histological examination of the small intestinal mucosa as obtained by the Crosby-Kugler capsule. Two patients were on a gluten free diet; one was on steroid therapy, the others being untreated.

Diagnosis of pernicious anæmia was made on finding megaloblastic anæmia associated with a low serum vitamin B_{12} level, achlorhydria on maximal histamine stimulation, and an abnormal Schilling test corrected by intrinsic factor. All patients responded to therapy with cyanocobalamin and most of the patients had been treated at the time of intubation. One patient had a small intestinal diverticulum.

Amongst the patients with intestinal loops there were four patients with jejunal diverticulosis and ten with loops in the distal small bowel. Most of these had impaired absorption of vitamin B_{12} though none had steatorrhœa. There was also a group of patients who had undergone gastric surgery.

Fifteen hospital control patients were studied, this group consisting of three normal people, two thyrotoxics, two neurotics, an obese patient, an epileptic, a patient with drug allergy, one with rheumatoid arthritis and four with testicular seminoma who had been treated surgically and given radiotherapy at least three years previously and had not relapsed.

Sampling at jejunal and ileal level

The method used was a nasal intubation technique. The tube was similar to that suggested by Blankenhorn, Hirsch and Ahrens (1955) and was of such a nature as to withstand autoclaving, while exposing the patient to the minimum discomfort and yet allowing adequate aspiration. Six

and a half foot lengths of polyvinyl tubing (Grade: E.R.P., Esco (Rubber) Ltd., London) with radio-opaque markers attached were used. The bore of the tube was 1·5 mm. and the external diameter 2·5 mm. Aspirating ports were cut in the region of the terminal 3 to 4 inches and a mercury bag attached to the end. All materials were autoclaved prior to intubation of the fasting patient in the morning when a fine plexitron tube was introduced nasally. The end of this was drawn out of the patient's mouth and to it the *proximal* end of the polyvinyl tube was attached with a sterile metal segment. Thus the polyvinyl tube could be drawn back out of the patient's nose, this being continued until, when the *distal* end with the mercury bag attached entered the mouth, the patient was allowed to swallow this. The proximal end of the polyvinyl tube, i.e. the site of attachment of the plexitron tube, was regarded as contaminated and cut off. Further lengths of polyvinyl tubing were attached by sterile metal segments as and when required.

The tube was washed through with 100 ml. of sterile saline after it had reached the stomach, this being repeated when the pylorus had been passed and again on completing the jejunal aspiration. Aspiration was carried out when the aspirating ports in the tube were calculated and assessed by fluoroscopy to be at mid-jejunal level, the initial aspirate being rejected. A similar procedure was carried out at mid-ileal level though delay in aspiration occurred here owing to the various times of the night at which the tube reached the desired site. There was always an interval of at least four hours between the time the tube had previously been washed through and the time of aspiration at jejunal or ileal level.

Sampling at gastric and jejunal level

Since the duration of intubation in these cases was shorter the oral route was used and fasting gastric juice aspirated. The procedure was then as already described but washing the tube was not repeated after it had entered the small intestine in the patients with gastric operations.

In all patients, passage through the pylorus was aided by turning them initially on to their right side. They were allowed a normal diet *throughout the experimental period* starting as soon as possible after intubation.

The aspirates were *immediately* made up into serial dilutions using nutrient broth. Viable counts were done on each specimen using the Miles and Misra technique (1938) of serial dilution with drop counts. The method would demonstrate the presence of organisms in as low a concentration as 250/ml. All estimations were performed in duplicate. In those patients undergoing jejunal and ileal aspiration it was hoped to isolate as many organisms as possible by using selective as well as ordinary media. Thus blood agar and MacConkey plates were cultured ærobically whilst blood agar, Willis and Hobbs' medium (1959) (for clostridia), Rogosa agar (Oxoid modification P.M. 221 of the formula of Rogosa, Mitchell and Wiseman, 1951) (for lactobacilli), neomycin blood agar (Smith and Crabb, 1961) (for *Bacteroides*) and thallous acetate agar plates (Barnes, 1956) (for anærobic streptococci) were cultured anærobically. The characteristics of individual colonies were noted and all colonies examined by Gram staining. *Clostridium welchii* were identified by the presence of opalescence (lecithinase production) and lactose fermentation around the colony.

In those patients undergoing gastric and jejunal aspiration, organisms of the fæcal type as represented by *Enterobacteriaceæ* and *Streptococcus fæcalis* were searched for on MacConkey agar. *Enterobacteriaceæ* were subdivided by their biochemical reactions to conform closely with the groups proposed by the Enterobacteriaceæ subcommittee of the International Committee on Bacterial Nomenclature and Taxonomy—Escherichia (Alkalescens-Dispar), Citrobacter, Klebsiella, Cloaca, Hafnia, Proteus, Providencia, Salmonella, Arizona, Shigella (Enterobacteriaceæ Subcommittee, 1958).

The ability of organisms to assimilate cyanocobalamin *in vitro* was estimated by preparing 10 ml. aliquots of a mixture of 150 ml. of Difco micro-inoculum broth to which 0·5 μg. of ^{58}Cobalt-labelled cyanocobalamin (Radiochemical Centre, Amersham) had been added. The solution was sterilised at 15 lb. pressure for 10 minutes. One ml. of a sixteen hour overnight culture of an organism was added to an aliquot. Two standards of 10 ml. of broth with added ^{58}Cobalt-labelled cyanocobalamin, but inoculated with 1 ml. sterile saline, were used as controls. After overnight incubation at 37°C. the material was centrifuged at 3,000 revolutions per minute for 30 minutes. The extent of radioactivity in the supernatant was calculated using a well type scintillation counter and compared with that in the uninoculated control samples. Measurement of radioactivity in the organisms confirmed that they had removed it from the culture medium. The radioactivity could not be washed off the organisms.

The rate of uptake of cyanocobalamin by the organisms was investigated by preparing 6 × 10 ml. of broth containing ^{58}Cobalt-labelled cyanocobalamin as above and adding to this 1 ml. of a fresh overnight culture of the organism under study. Centrifugation was done as above after incubation at zero time, one hour, two hours, five hours, twelve hours and twenty-four hours, and the activity of the supernatant calculated at each time.

Vitamin B_{12} absorption was calculated by the Schilling test (1953), modified in that carbachol, 0·25 mg., was given intramuscularly 15 minutes before the oral dose of labelled cyanocobalamin. Where required, the test was repeated with intrinsic factor or after a five day course of tetracycline in a dosage of 250 mg. four times daily.

RESULTS

Control patients

Table 1 shows the findings obtained on culturing the jejunal and ileal aspirates for the organisms previously mentioned in six hospital control patients. The striking feature is the absence of fæcal organisms in the upper small intestine. In two patients, i.e. 1 and 6, these organisms were present in the ileum.

TABLE 1

Bacteriological findings in the jejunum and ileum of six control patients

Case No.	Organisms found	Jejunal aspirate	Ileal aspirate	Diagnosis
1	Strep. viridans	3×10^5	—	Normal
	Staph. albus	1×10^6	—	
	Cl. welchii	—	1×10^4	
2	Lactobacilli	—	5×10^3	Hysterical neurosis
3	—	—	—	Rheumatoid arthritis
4	Strep. viridans	2×10^5	—	Thyrotoxicosis
	Staph. albus	1×10^6	—	
5	Diphtheroid bacilli	7×10^4	1×10^4	Hypochrondriasis
	Yeasts	—	1×10^4	
6	Strep. viridans	5×10^4	—	Obesity
	Coliform bacilli	—	4×10^6	

Results expressed as numbers of viable organisms per ml. aspirate.

In a further series of nine control patients who had their gastric and jejunal aspirates cultured only for fæcal type organisms, the organisms found were Citrobacter in a concentration of 5×10^2/ml. in the jejunum of a patient with treated testicular seminoma and *Streptococcus fæcalis* in the stomach of an epileptic and a normal person in concentrations of 2×10^3/ml. and 5×10^3/ml. respectively. As far back as 1919 Bessau and Bossert stated that they regarded as abnormal the presence of even a few coliform bacilli in the duodenal juice. We would agree with this and would classify a concentration of fæcal type organisms, as represented by the family *Enterobacteriaceæ* and *Streptococcus fæcalis*, of 10^4/ml. as abnormal in the upper small intestine.

Patients with malabsorptive disease

Table 2 shows the findings of full bacteriological investigation in the jejunum and ileum of eight patients with malabsorptive disease. As is seen, fæcal organisms in the upper small intestine were found in significant numbers in patients 18 and 19, in the latter the concentration of *Streptococcus fæcalis* only being significant. Yeast type organisms and lactobacilli were generally found only in small concentrations in the upper small bowel.

Patients 18 and 19 had quite a high concentration of *Streptococcus fæcalis* and patients 16, 17, 18, 19 and 20 of coliform organisms in the ileum. Lactobacilli and yeasts occurred, usually in low concentrations, in the ileum of most patients.

Patients with pernicious anæmia

In Table 3 there are illustrated the findings in fifteen patients with pernicious anæmia in whom the upper small bowel was investigated for fæcal type organisms. Patients 30, 34 and 38 had abnormal coliform counts in the stomach and upper

TABLE 2
Bacteriological findings in the jejunum and ileum of eight patients with malabsorptive disease

Case No.	Organisms found	Jejunal aspirate	Ileal aspirate
16	Lactobacilli	—	4×10^4
	Coliform bacilli	—	2×10^7
	Yeasts	4×10^2	1×10^6
	Anærobic streptococci	—	4×10^5
17	Lactobacilli	—	4×10^3
	Coliform bacilli	—	1×10^7
	Yeasts	2×10^3	1×10^4
18	Lactobacilli	1×10^4	4×10^4
	Strep. fæcalis	4×10^5	3×10^5
	Coliform bacilli	4×10^4	3×10^6
	Cl. welchii	—	2×10^3
19	Strep. viridans	6×10^5	1×10^6
	Staph. albus	3×10^5	—
	Neisseria	2×10^3	—
	Lactobacilli	1×10^3	2×10^3
	Strep. fæcalis	3×10^5	2×10^7
	Coliform bacilli	2×10^3	4×10^6
20	Strep. viridans	2×10^4	—
	Neisseria	1×10^4	—
	Lactobacilli	—	3×10^4
	Coliform bacilli	—	1×10^8
	Yeasts	—	6×10^5
21	Strep. viridans	3×10^3	1×10^3
	Yeasts	3×10^3	5×10^2
22	Strep. viridans	7×10^3	4×10^2
	Lactobacilli	3×10^3	1×10^3
	Yeasts	4×10^3	4×10^4
23	Lactobacilli	1×10^4	4×10^3
	Yeasts	4×10^2	1×10^3

Results expressed as numbers of viable organisms per ml. aspirate.

TABLE 3
Fæcal type flora in stomach and jejunum of fifteen patients with pernicious anæmia

Case No.	Organisms found	Gastric aspirate	Jejunal aspirate
24	—	Not done	—
25	—	Not done	—
26	—	Not done	—
27	—	Not done	—
28	—	—	—
29	—	—	—
30	Escherichia	1×10^8	1×10^8
31	—	—	—
32	—	—	—
33	—	—	—
34	Escherichia	7×10^6	3×10^6
	Proteus	—	1×10^7
35	Escherichia	—	4×10^3
	Strep. fæcalis	—	2×10^6
36	Strep. fæcalis	1×10^3	5×10^3
37	Strep. fæcalis	—	2×10^5
38	Alk. dispar	4×10^6	2×10^7

Results expressed as numbers of viable organisms per ml. aspirate.

small intestine. Patients 35 and 37 had an abnormal concentration of *Streptococcus fæcalis* in the upper small intestine. In the others there was no significant difference from the findings in the control group.

Table 4 shows the results of the more extensive bacteriological studies at jejunal and ileal levels in five of these patients (cases 24 to 28). It will be seen that *Clostridium welchii* were absent from the upper small intestine. In two patients *Clostridium welchii* were found in the ileum and patient 28 had coliforms in the same region.

Patients with blind or stagnant loops

Table 5 shows the bacteriological findings of fæcal type flora in four patients with small intestinal diverticula. In patients 39 and 40 the flora was abnormal. Absorption of vitamin B_{12} was also abnormal and could be corrected by antibiotics in these patients. In the two other patients the flora was abnormal in one (case 42) and the absorption of vitamin B_{12} normal in both.

TABLE 4

Bacteriological findings in the jejunum and ileum of five patients with pernicious anæmia

Case No.	Organisms found	Jejunal aspirate	Ileal aspirate
24	Strep. viridans	1×10^6	1×10^4
25	Strep. viridans	2×10^6	4×10^4
	Staph. albus	2×10^6	5×10^6
	Lactobacilli	5×10^2	4×10^3
26	Strep. viridans	2×10^6	—
	Staph. albus	1×10^6	—
	Cl. welchii	—	1×10^4
27	—	—	—
28	Strep. viridans	2×10^6	5×10^5
	Staph. albus	3×10^3	—
	Lactobacilli	—	4×10^4
	Coliform bacilli	—	6×10^6
	Cl. welchii	—	5×10^5

Results expressed as numbers of viable organisms per ml. aspirate.

TABLE 5

Fæcal type flora in stomach and jejunum of four patients with jejunal diverticula

Case No.	Organisms found	Gastric aspirate	Jejunal aspirate	Vit. B_{12} absorpt.	Presentation
39	Klebsiella	—	1×10^7	Abnormal	Iron deficiency anæmia
40	Escherichia	Not done	5×10^8	Abnormal	Gastrointestinal symptoms
41	Escherichia	4×10^3	5×10^2	Normal	Gastrointestinal symptoms
	Strep. fæcalis	8×10^3	—	—	
42	Escherichia	—	1×10^7	Normal	Gastrointestinal symptoms

Results expressed as numbers of viable organisms per ml. aspirate.

Table 6 illustrates the findings in ten patients with surgical blind loops (mainly ileo-transverse colostomies) involving the lower small bowel. An abnormal jejunal flora was present in patients 44, 45, 46 and 47. Though the small intestinal findings in case 43 were normal, the gastric coliform count may be significant. These patients had impaired absorption of vitamin B_{12} which was corrected or improved by antibiotics in cases 43, 44, 45 and 46.

In only case 51, who had an unknown length of ileum resected with an end to side anastomosis, was absorption corrected by intrinsic factor.

In addition to *Enterobacteriaceæ* and *Streptococcus fæcalis* we have looked for *Clostridium welchii* in six patients with blind or stagnant loops, but did not find this organism to be present in the jejunum.

TABLE 6

Fæcal type flora in stomach and jejunum of ten patients with loops in the lower small bowel and impaired absorption of cyanocobalamin

Case No.	Organisms found	Gastric aspirate	Jejunal aspirate	Vit. B_{12} absorpt. after tetracycline	Diagnosis
43	Escherichia	3×10^4	—	Improved	Ileo-transverse colostomy for appendicitis
44	Strep. fæcalis	2×10^3	1×10^3	Corrected	Ileo-transverse colostomy for appendicitis
	Escherichia	—	3×10^3		
	Klebsiella	1×10^3	—		
	Proteus	—	2×10^4		
45	Escherichia	—	2×10^7	Corrected	Ileo-transverse colostomy for appendicitis
46	Escherichia	Not done	4×10^6	Corrected	Ileo-transverse colostomy for appendicitis
47	Klebsiella	Not done	5×10^6	Abnormal	Ileo-transverse colostomy for Crohns Colonic cancer
48	—	—	—	Abnormal	Ileo-transverse colostomy for Crohns
49	—	—	—	Abnormal	Ileo-transverse colostomy for appendicitis
50	Strep. fæcalis	6×10^3	—	Abnormal	Ileo-transverse colostomy for adhesions
51	—	—	—	Abnormal	Ileal resection Pernicious anæmia
52	Escherichia	2×10^3	2×10^3	Not done	Ileo-transverse colostomy for Crohns Gastro-enterostomy

Results expressed as numbers of viable organisms per ml. aspirate.

Patients with gastric operations

The results of examining the upper bowel content for fæcal type organisms in patients with gastric operations are found in Table 7. It was not unusual to be able to isolate coliform organisms from the gastric and jejunal contents of this group and in seven cases the number of organisms in the jejunum was in the abnormal range (Nos. 53, 54, 55, 58, 62, 65 and 66). *Clostridium welchii* have been looked for at these levels in six of these patients but not found.

Studies in vitro

Studies of the uptake of labelled cyanocobalamin were carried out with thirty-five strains of organisms, and the results are shown in Table 8.

These results confirm previous findings (Girdwood, 1955 *b*; 1959 ; Doig and Girdwood, 1960). It will be seen that the *Proteus* group of organisms are less active in their ability to remove vitamin B_{12} from the culture medium than are other organisms of the family *Enterobacteriaceæ*. Strains of *Streptococcus fæcalis* took up very little labelled cyanocobalamin.

It is seen from Figs. 1, 2 and 3 that, when the rates of uptake of the vitamin were measured, on an average an organism required about five hours to attain its maximum uptake under the conditions of the investigation. The pattern of uptake by organisms isolated from patients with blind or stagnant loops was similar to

TABLE 7

Fæcal type flora in stomach and jejunum of fifteen patients with gastric operations

Case No.	Organisms found	Gastric aspirate	Jejunal aspirate	Vit. B_{12} absorpt.	Steatorrhœa	Operation
52	Escherichia	2×10^3	2×10^3	Not done	No	Gastro-enterostomy
53	Escherichia	8×10^7	8×10^4	Abnormal	No	Gastro-enterostomy
54	Escherichia	3×10^4	8×10^6	Abnormal	Yes	Gastro-enterostomy
55	Citrobacter	—	2×10^4	Normal	No	Gastro-enterostomy
56	—	—	—	Normal	No	Gastro-enterostomy
57	Citrobacter	—	2×10^3	Normal	No	Gastro-enterostomy
58	Escherichia	—	9×10^5	Normal	No	Partial gastrectomy
59	Proteus	2×10^3	8×10^3	Abnormal	No	Partial gastrectomy
60	—	—	—	Abnormal	Not done	Partial gastrectomy
61	—	—	—	Normal	No	Partial gastrectomy
62	Escherichia Citrobacter	1×10^7 5×10^2	6×10^6 8×10^5	Abnormal	No	Partial gastrectomy
63	Escherichia	2×10^4	3×10^3	Abnormal	No	Partial gastrectomy
64	Escherichia Strep. fæcalis	6×10^4 3×10^3	3×10^3 —	Abnormal	No	Partial gastrectomy
65	Klebsiella	4×10^4	4×10^4	Abnormal	No	Partial gastrectomy
66	Escherichia	2×10^5	1×10^6	Abnormal	No	Partial gastrectomy

Results expressed as numbers of viable organisms per ml. aspirate.

TABLE 8

Uptake of labelled cyanocobalamin by organisms isolated from the G.I. tract

	Organism genus	No. of strains	Mean % uptake of radio-activity	Range
1.	Escherichia	13	88·5%	78–98%
2.	Klebsiella	5	87%	80–93%
3.	Alkalescens-dispar	3	87%	80–91%
4.	Citrobacter	1	84%	
5.	Cloaca	1	84%	
6.	Proteus*	3	70%	56–83%
7.	Streptococcus fæcalis	9	6%	0–20%

* Incl. Providencia.

that of organisms isolated from other patients. The number of *Proteus* strains isolated was only three, and six organisms obtained from the National Collection of Type Cultures (Colindale Avenue, London, N.W.9) were therefore examined (Fig. 4). The curves confirm that these organisms are less active in their uptake of cyanocobalamin.

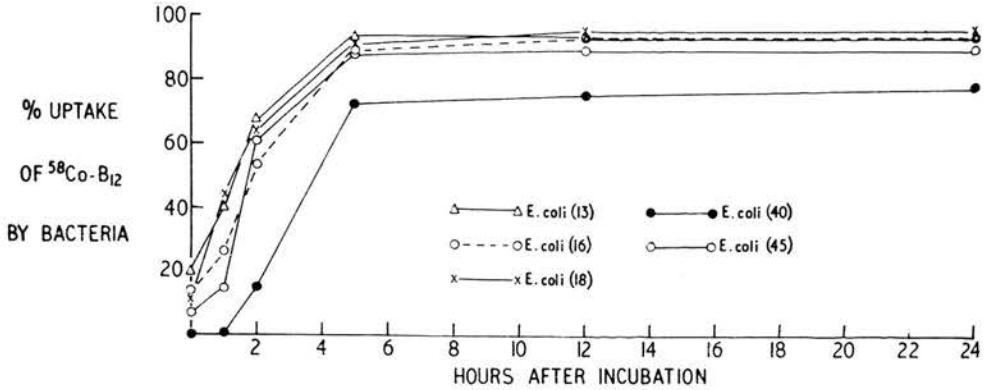

Fig. 1. *Uptake of labelled cyanocobalamin by intestinal bacteria.* Numbers in brackets are authors' reference to strain of organism.

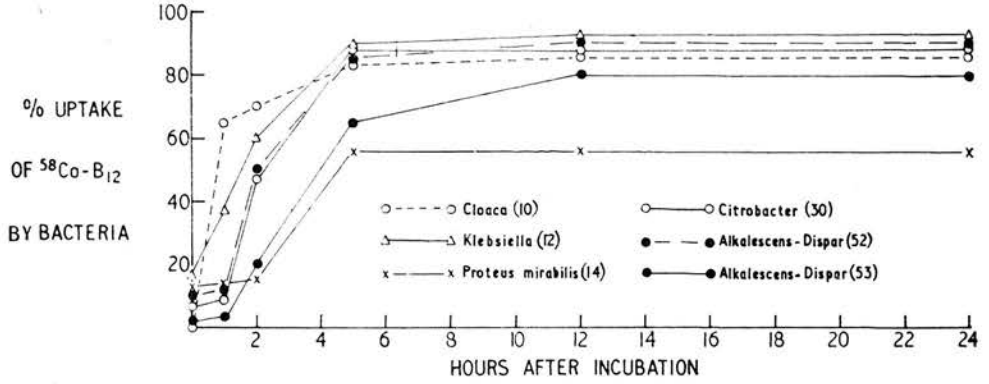

Fig. 2. *Uptake of labelled cyanocobalamin by intestinal bacteria.* Numbers in brackets are author's reference to strain of organism.

Discussion

Method of sampling

As French (1961) points out, the inaccessibility of the small intestine and the rapid alteration of its contents during digestion make it very difficult to characterise what is taking place where gut flora is concerned. The direct sampling technique (Blacklock, Guthrie and Macpherson, 1937; Cregan and Hayward, 1953) is attractive but allows only semi-quantitative estimation of organisms, and the state of the bowel in the fasting anæsthetised patient is not necessarily the same as in the ambulant patient. Previous intubation studies have generally involved the use of large tubes, including Miller Abbott tubes, and most studies have dealt with patients in the fasting state (French, 1961).

It is felt that the tube chosen for the present work has some advantages. It causes little discomfort and our patients were able to partake of a normal ward diet with no difficulty throughout the period of intubation. The aspiration of gastric juice, however, was done in the fasting state immediately following intubation. The smaller tube, with its marked pliability at body temperature, is perhaps less likely than a larger tube to interfere with small intestinal function, and in particular the " creeping " up the tube of the small bowel, which adds to the difficulty of estimating the level at which one is aspirating, is likely to be less.

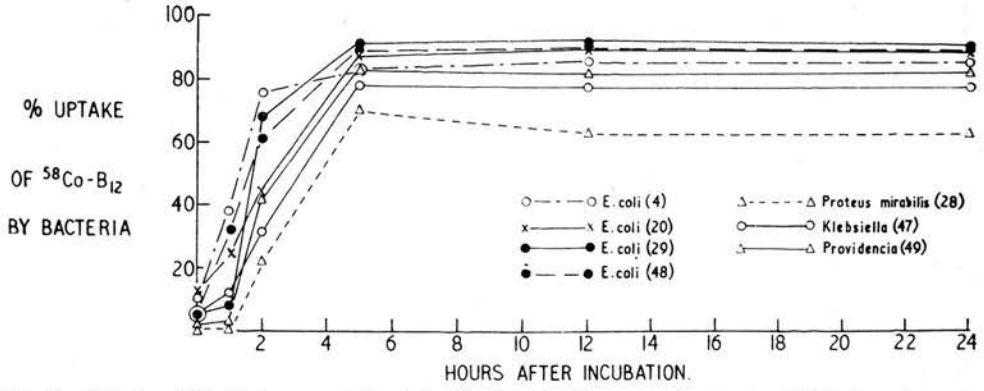

Fig. 3. *Uptake of labelled cyanocobalamin by the intestinal bacteria of patients with blind or stagnant loops.* Numbers in brackets are authors' reference to strain of organism.

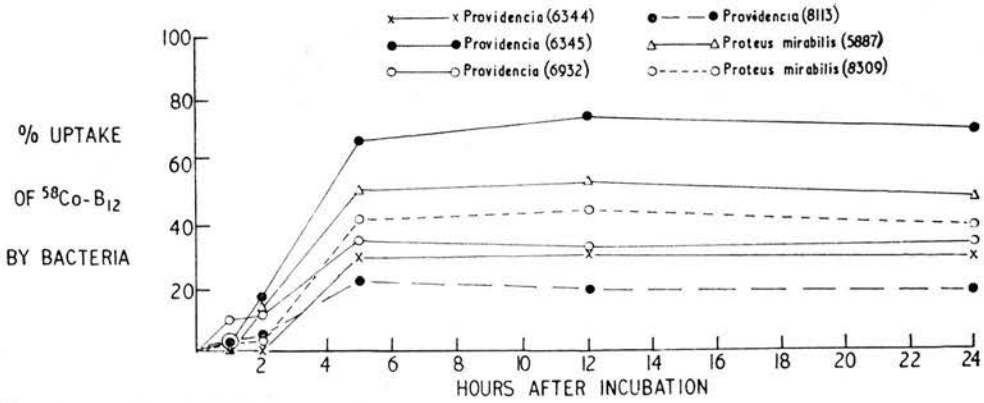

Fig. 4. *Uptake of labelled cyanocobalamin by control series of proteus organisms.* Numbers in brackets refer to the catalogue number of the strain. (Catalogue of Species. M.R.C. Memorandum No. 35.)

However, the use of an intubation technique results in many oropharangeal organisms being carried down by the tube and, for this reason, little comment is made about the finding of such flora. The isolation of a flora of the fæcal type is of more significance since it is unlikely to have been carried down by the tube. Considerable simplicity is also introduced, as one can with reasonable justification classify a fæcal flora by the family *Enterobacteriaceæ* and the genus *Streptococcus fæcalis*.

Control patients

The virtual absence of these fæcal organisms in the normal small intestine at jejunal level is in agreement with the findings of Blacklock *et al.* (1937) and Cregan and Hayward (1953). It will be noticed that one of the controls in our study had coliform organisms present in the ileum and another some *Clostridium welchii* (Table 1).

By intubation Nichols and Glenn (1940) found coliforms to be unusual in the ileum of normal controls. Blacklock and his co-workers found coliform organisms in the lower ileum of one third of his young patients, though the concentrations

are not stated. Cregan and Hayward found them in three of fourteen patients, in two of these in significant concentrations. Unlike in the present study, all the above were done in fasting subjects. The finding of a fæcal flora at ileal level is therefore difficult to interpret, and it would seem that the demonstration of this type of flora is likely to be of more significance, the higher up the small bowel that it is found.

These considerations led to the curtailing of the earlier more extensive bacteriological studies to the study of a fæcal type flora and, in particular, in most of the patients to the study of samples obtained from the jejunum.

Patients with malabsorptive disease

Bearing in mind the difficulty in interpreting the findings of oropharangeal type flora it is seen that in these patients the upper small intestinal flora differs little from those in the control group of patients. As in the controls fæcal flora is absent from the jejunum in most of the patients. *Streptococcus fæcalis* appears in abnormal concentrations in the jejunum of patients 18 and 19, and we would also classify the coliform count in patient 18 as just abnormal (Table 2). This last patient was on steroid therapy and other patients on these drugs are at present being studied to see if such therapy is associated with an abnormal small bowel flora. It is difficult to explain the concentration of *Streptococcus fæcalis* in case 19. It may be relevant that patients 18 and 19 were the most ill in this series.

It will be seen that several patients had coliform organisms in the ileum. In spite of the previous remarks regarding the difficulty of interpreting fæcal flora at this site, it may be that the incidence of these organisms in this group of patients is greater than would be expected.

All patients except Nos. 22 and 23 showed impaired absorption of vitamin B_{12} but it was impossible to draw any conclusions about the relationship between this and the presence of *Enterobacteriaceæ* in the small bowel. Certainly in patients 17 and 18 broad spectrum antibiotics did not improve absorption of the vitamin.

In the search for specific organisms as the cause of malabsorptive disease, it has been suggested that lactobacilli or yeasts are responsible. It will be noticed that in the group of patients investigated here these organisms were not infrequently isolated from the small bowel. It is not suggested that this indicates that they are responsible for the disease and, in fact, in the low concentrations found they are very likely to be a secondary phenomenon, as previously suggested (French, 1961).

Patients with pernicious anæmia

The results obtained in our patients indicate that so far as fæcal flora is concerned there is no demonstrable difference between the findings in the majority of patients with pernicious anæmia from the control patients. In patient 34 (Table 3) the increase in coliform organisms may have been due to the presence of a diverticulum in the upper small intestine. Radiological examination of the small intestine in cases 30 and 38, both of whom had an abnormal fæcal type flora in the jejunum, revealed no local abnormality which might explain the findings. Both these patients were in complete remission at the time of study. In the light of the findings in the control patients the concentration of *Streptococcus fæcalis* in patients 35 and 37 is also abnormal. It appears therefore that in some patients with pernicious anæmia there is an increase of fæcal type organisms in the jejunum.

With regard to the ileal contents in these patients, one had some coliforms present in this region and two had *Clostridium welchii* (Table 4). In view of the findings in the control group (Table 1) this should not be interpreted as being necessarily of any significance.

Many observers have emphasised the existence of an abnormal flora in the small intestine of patients with pernicious anæmia and much of the literature prior

to 1930 is reviewed by Moench, Kahn and Torrey (1925) and Davidson and Gulland (1930). However, the descriptions of the abnormal flora in patients with pernicious anæmia have been based on the findings in the gastro-duodenal aspirates and fæces, and not on the findings in the small intestine itself. Thus Davidson and Gulland found significant numbers of coliform organisms in the " gastro-duodenal contents " of thirteen out of twenty patients with this condition. Of the eleven patients in whom we examined gastric contents, three had significant coliform counts present. We, unlike these authors, were studying the fasting gastric juice. Although the view that the features of the disease are attributable to the absorption of a toxin from the alimentary tract is not now accepted, it is still a hypothesis that an abnormal small intestinal flora exists, particularly as regards fæcal organisms. The high pH of the gastric and therefore the intestinal contents is invoked as being responsible for this (Davidson and Gulland, 1930) though the findings of Cregan, Dunlop and Hayward (1953) in patients with low gastric acidity indicated that the flora of the small intestine was independent of acid secretion by the stomach.

Patients with pernicious anæmia have been reported as having a markedly increased concentration of *Clostridium welchii* in the fæces (Moench *et al.*, 1925). The reason for this is obscure, but Davidson and Gulland (1930) thought that it must be due to an increase of spores in the fæces of these patients as the vegetative forms of the organisms which were present were found to be dead. They suggested that the " altered biochemical reaction of the contents of the small intestine " in pernicious anæmia might be largely responsible for the findings in the fæces. Moench and her co-workers (1925) supported the opinion of previous observers in suggesting that this finding indicated an increase in the concentration of these organisms in the small intestine of patients with pernicious anæmia. Davidson and Gulland were unable to demonstrate any significant increase in the number of Clostridia in the gastro-duodenal contents of their patients with this disease and the present work has shown that these organisms were absent from the upper small intestine of the patients studied (Table 4).

In general, it may be said that the present findings do not support the contention of previous authors that, in patients with pernicious anæmia, the small intestinal content of fæcal organisms consistently differs from that of controls and this supports the observations of Cregan *et al.* (1953) that the manner in which the small intestine is kept relatively free of these organisms is independent of gastric acidity.

Patients with blind or stagnant loops

It will be seen that in our patients with blind or stagnant loops the finding of an abnormal flora was not infrequent. These patients not uncommonly exhibit evidence of impaired absorption of cyanocobalamin and it has been suggested that this is due to intestinal organisms which compete with the host for the vitamin (Halsted, Lewis and Gasster, 1956; Doig and Girdwood, 1960).

It has been demonstrated that organisms of the family *Enterobacteriaceæ*, the predominant organism in these loops, are active as regards their uptake of vitamin B_{12} *in vitro* (Table 8). The concept of bacterial competition for vitamin B_{12} in patients with blind or stagnant loops is, however, the subject of some controversy. It is known that various substances, including gastric juice, will bind cyanocobalamin rendering it unavailable to micro-organisms that would otherwise take it up *in vitro* (Ternberg and Eakin, 1949; Bird and Hoevet, 1951; Burkholder, 1952; Hoff-Jørgensen, 1952; Drexler, 1958). Since under physiological conditions vitamin B_{12} is present in the small intestine bound to intrinsic factor it has been suggested that the vitamin is not available to the organisms *in vivo*. It is of interest to note here that Spray (1952) was of the opinion that the factor in human gastric juice which bound vitamin B_{12} so that it was not available to micro-organisms *in vitro* was not identical with intrinsic factor since the latter was much more thermolabile

than the former. Gräsbeck (1956, 1960) was of the view that they probably are identical. More recently Booth and Heath (1962) have shown that, though *Escherichia coli* are capable of inhibiting absorption of vitamin B_{12} when these are fed together to the rat, the inhibitory effect of the organisms was partially abolished when the vitamin had previously been exposed to rat gastric extract. They therefore suggest that micro-organisms in these patients may not inhibit absorption by competitive uptake and as an alternative hypothesis postulate that organisms may interfere in some way with the transport mechanism in the distal small intestine.

These various findings are difficult to interpret in relation to the living body because the number of bacteria in the small intestine may vary from hour to hour and because we do not know how gastrointestinal secretions affect the interactions between micro-organisms and vitamin B_{12} in the intestine. Before assuming, however, that the experiments *in vitro* mean that vitamin B_{12} is rendered unavailable to micro-organisms in the gastrointestinal tract of man, it must be said that several workers have shown that the intensity of binding of vitamin B_{12} by gastric juice is not necessarily correlated with intrinsic factor activity and that preparations which are potent with respect to intrinsic factor may bind the vitamin in an easily dissociable form (Raine, 1955; Gräsbeck, 1956). Indeed it has been found that all the vitamin B_{12} bound to gastric juice was accessible to adsorption by *Lactobacillus leichmannii* if the pH was less than 4, though the same could not be shown with *Escherichia coli* (Gräsbeck, 1957).

With these points in mind, therefore, we have attempted to apply our results *in vitro* to the findings *in vivo* though obviously this must be done with caution.

The studies *in vitro* on the rates of uptake by organisms of the family *Enterobacteriaceæ* have demonstrated that under the conditions of our experiments an organism requires about five hours to attain its maximum uptake of the vitamin. Since food takes about four hours from the time of intake to reach the large intestine (Starling and Lovatt-Evans, 1962), it is not difficult to imagine how blind or stagnant loops in the *proximal* small bowel are able to flood this region with organisms which might bind the vitamin and thus render it unavailable for absorption at the terminal ileum. Indeed in the two patients with jejunal diverticula and impaired absorption of cyanocobalamin, which was correctable by antibiotics, the coliform concentration in the jejunal juice was high (Table 5, Nos. 39 and 40). The five hour lapse *in vitro* before maximum uptake of the vitamin makes it more difficult to understand how, if these results are relevant to what happens in the body, a blind or stagnant loop in the *distal* reaches of the small bowel could allow its inhabiting organisms time to bind the vitamin rendering it unavailable for absorption.

In this connection the findings expressed in Table 6 are of interest. All these patients had undergone similar bowel operations and with one exception, who was not available for absorption studies (No. 52), all exhibited impaired absorption of cyanocobalamin. Antibiotic therapy corrected or markedly improved absorption in patients 43, 44, 45 and 46 and, with the exception of case 43, coliform organisms were found in higher than normal concentrations in the upper small intestine. The gastric coliform count in patient 43 may be significant. An abnormal flora has been described recently (Bishop, 1963) in the small intestine both proximal and distal to the site of surgically fashioned blind loops in dogs. Our findings demonstrate an abnormal flora well proximal to the site of the loop in man.

Antibiotics did not affect the absorption of cyanocobalamin in patients 47, 48, 49, 50 and 51 and only in this last mentioned case was absorption corrected by intrinsic factor. Except for case 51, therefore, the impaired absorption of cyanocobalamin in these patients was due to disease or bypassing of the terminal ileum. In these cases coliform organisms were not found in abnormal numbers in the

upper small intestine, except in one case. The one exception, case 47, had subacute intestinal obstruction due to a malignant tumour of the ascending colon. Bishop and Allcock (1960) have found that patients with intestinal obstruction can exhibit a very abnormal flora in the small intestine and this probably accounts for the abnormal findings in this case.

From these findings, therefore, it would seem that patients with blind or stagnant loops in the distal small bowel and impaired absorptions of vitamin B_{12} can be divided into two groups. There are those with an abnormal content of fæcal type organisms in the upper small intestine and those without this. In the former group absorption of vitamin B_{12} is correctable by antibiotic therapy and in the latter group it is not. It is likely that a diseased or bypassed ileum is responsible for the impaired absorption of the vitamin in the second group, though using the tests available to us at present it is not possible to say with certainty that the stagnant loop present in these patients does not also operate.

It will be seen therefore that a blind loop in the *distal* small bowel giving rise to impaired absorption of vitamin B_{12} is associated with an abnormal concentration of coliform organisms in the upper small intestine. This obviously brings the organism into contact with the vitamin at an early stage during its passage down the small bowel. If our findings *in vitro* have any bearing on what happens *in vivo*, then in view of the five hours delay *in vitro* between availability and maximum uptake of the vitamin, obviously the higher the organisms are in the small intestine, the more likely it is that they may bind the vitamin before it reaches its site of absorption in the ileum.

General points

It would be of great interest to try to establish the source of the abnormal fæcal flora in the upper small intestine which occurs in some patients. One should resist the temptation to put it down to growth from below and indeed the observations of Bishop and Allcock (1960) would seem to indicate that the abnormal flora is obtained from ingestion, at least in patients with intestinal obstruction. What exactly prevents these organisms from establishing a hold in the upper reaches of the small intestine in normal circumstances it is impossible to say. Peristaltic activity is probably of importance and the recent animal experiments in which drug induced paralytic ileus was associated with the presence of an abnormal flora in the small intestine would support this view (Dixon and Paulley, 1963). Thus it is worth recalling the observations of Howie and his colleagues on the findings of clostridia in the stomachs of patients recently exposed to gastric operations (Howie, Duncan and Mackie, 1953; Duncan, Goudie, Mackie and Howie, 1954). It is possible that we are constantly ingesting fæcal type organisms in low concentrations and that these remain undetected until some change in the pattern of bowel function allows their multiplication. In this context previous authors have used the terms " transient " and " permanent " flora—the latter description being given to organisms which are present in such concentration that they can be said to be capable of multiplication in the part of the intestine sampled (Cregan and Hayward, 1953). Using the present technique, the finding of fæcal type organisms in a concentration of 10^4/ml. has been defined as abnormal but we are reluctant to use the word " permanent ". Thus the finding of fæcal type organisms in the upper small bowel in patients with stagnant or blind loops in the proximal bowel following gastric operations was not unusual (Table 7). Using the Schilling test it is impossible to interpret the significance, if any, of the organisms in impaired absorption of vitamin B_{12} when the defect is apparently corrected by intrinsic factor as in patients 53, 54, 62, 65 and 66. However, in cases 55 and 58 vitamin B_{12} absorption was normal in spite of abnormal concentrations of coliforms in the upper small intestine. It may be that the bacteriological findings in this group of patients merely reflect

evidence of a transient phenomenon such as the discharge of proximal loop content into the small intestine just prior to sampling. This may also explain the abnormal counts in the patient with pernicious anæmia and an intestinal diverticulum (Table 3, case 34).

It is impossible to compare the findings in these patients with those of Goldstein, Wirts and Kramer (1961) and Wirts and Goldstein (1963) who investigated the flora of patients with partial gastrectomies and related their findings to the stool fat content of the patients. Too few of our patients had steatorrhœa to make such a study possible.

It is felt that the presence of fæcal type flora in the upper small intestine must be interpreted with caution and it would seem that a high intestinal aspirate containing an abnormally high concentration of coliform organisms, may be evidence of either a transient or permanent invasion of the upper small intestine and can only be interpreted in the light of knowledge of intestinal anatomy and retrospectively in the light of the clinical and investigative findings. Thus, for example, the absence of fæcal type organisms in the upper small intestine of a patient with a blind or stagnant loop which was giving rise to impaired absorption of cyanocobalamin, regardless of the site of this loop in the small intestine, would be surprising. However, the presence of such a flora even in obviously high concentrations is not diagnostic of the presence of such a loop.

SUMMARY

1. The intestinal flora has been studied in man by an intubation technique using a fine polyvinyl tube. The flora was studied in a control group of fifteen patients, eight others with malabsorptive disease, fifteen with pernicious anæmia and in twenty-eight with congenital or acquired blind or stagnant loops in the small intestine; this latter group included patients with jejunal diverticula, some with ileo-transverse colostomies and others who had undergone gastric operations.

2. The finding of lower bowel organisms in the normal small intestine was unusual. In patients with malabsorptive disease the small intestinal flora did not differ consistently from that of the control patients though yeasts and lactobacilli did appear to be more common. The flora in the small intestine of the majority of patients with pernicious anæmia was not significantly different from that of the control patients.

3. In patients with blind or stagnant loops in the small bowel the finding of an abnormal flora was not infrequent. The degree and rate of uptake of radioactive vitamin B_{12} by the organisms of the coliform group which were isolated was investigated. An attempt was made to relate these findings *in vitro* to the result of vitamin B_{12} absorption in the same subjects.

4. The significance of abnormal flora in the small intestine is discussed.

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BACTERIOLOGY OF THE SMALL INTESTINE IN NORMAL INDIANS

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The possible relevance of an abnormal flora in the small intestine of man to disease, particularly malabsorptive conditions, has been the subject of study for many years. The flora has been investigated mainly by various intubation techniques though the method of Cregan and Hayward (1953) which involves needle biopsy of the intestine at laparotomy, has been used by various observers. As French (1961) pointed out, one important omission of the work to date has been that the patient studied have been in the fasting state and this may have influenced the findings. We have previously reported the investigation of several groups of patients by means of an intubation technique which allows patients to take their normal diet (Dellipiani and Girdwood, 1964). The present paper describes the findings in a group of Indians with no known disease of the gastrointestinal tract, studied under Indian environmental and dietary conditions.

Material and Method

The subjects were investigated during a visit to the Medical College of Baroda in Gujarat State in India. A total of sixteen subjects were successfully studied. Seven of these were patients

in hospital who had no complaint relevant to the gastrointestinal tract, and were taking a normal ward diet, all were strict vegetarians. The rest of the subjects were mainly students and most of them took a mixed diet.

The methods used have been described fully elsewhere (Dellipiani and Girdwood, *loc. cit.*). This employs the use of an autoclaved polyvinyl tube of bore 1·5 mm. and external diameter 2·5 mm. Samples were taken from the stomach after an overnight fast, the patient then taking a normal breakfast. Normal meals were allowed subsequently and aspiration was repeated when it was assessed radiologically that the terminal aspirating segment was in the jejunum.

Aspirates were immediately made up into serial dilutions and viable counts done on each specimen using the method of Miles *et al.* (1938), all estimations being performed in duplicate. Organisms of the faecal type as represented by Enterobacteriaceae and *Streptococcus faecalis* and *Clostridium welchii* were looked for using MacConkey agar plates cultured aerobically and bood agar plates cultured anaerobically. Further identification of organisms found was done using routine bacteriological techniques. Enterobacteriaceae were subdivided to conform closely with the groups proposed by the Enterobacteriaceae Subcommittee of the International Committee on Bacterial Nomenclature and Taxonomy (Enterobacteriaceae Subcommittee, 1958). As before (Dellipiani and Girdwood, *loc. cit.*) the finding of a concentration of 10^4/ml. or more of the organisms looked for in the upper small intestine is defined as abnormal since in normal patients studied in Edinburgh the number of these organisms do not exceed this concentration at such a level.

Results

The results in twelve normal subjects are illustrated in Table 1. With the exception of

patient No. 10, who had a concentration of coliform organisms in the jejunum of 8×10^7/ml., all the findings were within normal limits. This patient was on benzhexol therapy for Parkinson's disease and it was felt that this drug might be influencing the bacterial content of the small bowel by some effect on gastrointestinal motility. Ten further subjects were therefore studied on the sixth day of a course of propantheline bromide 15 mg. q.i.d. Presumably owing to the effects of this drug on gastrointestinal motility, the intubation was completed successfully on only four subjects (three students and one patient). The results are illustrated in Table 2. Again it will be seen that the findings are normal.

TABLE 1—SHOWING BACTERIOLOGICAL FINDINGS IN JEJUNUM AND ILEUM OF TWELVE CONTROL SUBJECTS

Patient No.	Organisms found	Gastric aspirate	Jejunal aspirate	Condition	Diet
1	—	—	—	Doctor	Vegetarian
2	—	—	—	Technician	Vegetarian
3	Citrobacter	—	2×10^3	Student	Mixed
4	—	—	—	Student	Mixed
5	—	—	—	Student	Mixed
6	—	—	—	Student	Mixed
7	—	—	—	Thyrotoxicosis	Vegetarian
8	—	—	—	Muscular dystrophy	Vegetarian
9	—	—	—	Pharyngitis	Vegetarian
10	Escherichia	6×10^3	8×10^7	Parkinson's disease	Vegetarian
11	Escherichia	—	8×10^3	No diagnosis	Vegetarian
12	—	—	—	Mitral stenosis	Vegetarian

TABLE 2—SHOWING BACTERIOLOGICAL FINDINGS IN JEJUNUM AND ILEUM OF FOUR CONTROL SUBJECTS ON PROPANTHELINE BROMIDE

Patient No.	Organisms found	Gastric aspirate	Jejunal aspirate	Condition	Diet
1	—	—	—	Student	Vegetarian
2	Escherichia	—	3×10^3	Student	Mixed
3	—	—	—	Student	Mixed
4	Escherichia	3×10^3	—	Pneumothorax	Vegetarian

DISCUSSION

The results indicate that in the subjects studied the flora of the small intestine does not differ from that reported in Europeans using the same technique with one exception, viz., patient No. 10. The findings here were so unusual that in addition to considering the treatment he was receiving, radiological investigations were undertaken to exclude the possibility that some local lesion might have been responsible for the finding. In fact a jejunal diverticulum of moderate size was found. The possibility that his treatment with benzhexol might be responsible for the abnormal findings would seem unlikely in view of our results on volunteers on propantheline bromide, though of course the possibility cannot be excluded. Propantheline bromide was chosen for the study because it is known via its atropine like activity to slow bowel peristalsis (Texter and Barborka, 1954) and it was thought that the influence, if any, of benzhexol would be mediated in a similar manner. On the whole we feel that the single jejunal diverticulum was probably responsible for the abnormal bacteriological findings and in fact we have suspected a single diverticulum of being responsible for similar findings previously (Dellipiani and Girdwood, *loc cit.*, patient No. 34).

Summary

The intestinal flora has been studied in a group of Indian subjects in India with no known disease of the gastrointestinal tract, who were allowed a normal diet throughout the study. This was carried out by an intubation technique using a fine polyvinyl tube. The findings do not differ from the previously reported normal findings in a group of European subjects studied by the same method.

Acknowledgment

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The Significance of Abnormal Bacterial Proliferation in the Gastrointestinal Tract after Gastric Surgery

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Abstract: DELLIPIANI, A. W. & GIRDWOOD, R. H. The Significance of Abnormal Bacterial Proliferation in the Gastrointestinal Tract after Gastric Surgery. *Scand. J. Gastroent.* **2**, 161-168, 1967. The juices of the stomach or stomach remnant and of the jejunum of twenty-one patients who had undergone gastroenterostomy and twenty-six patients with partial gastrectomy have been studied for the presence of faecal type organisms as represented by *Enterobacteriaceae*, *Streptococcus faecalis* and *Clostridium welchii*. In the postgastrectomy cases faecal type organisms were commonly found in abnormal concentrations. After gastroenterostomy with vagotomy the bacteriological findings were very similar. It was not possible to show any relationship between the presence of steatorrhoea, diarrhoea, and impaired absorption of vitamin B_{12} and the finding of abnormal numbers of these organisms. The possible effect on the bacteriological findings of the achlorhydria which was commonly found in these patients is discussed.

Index-words: Gastrointestinal system; stomach; gastrectomy; bacteriology

The long term complications that follow gastric surgery are numerous and include steatorrhoea, diarrhoea, and impaired absorption of vitamin B_{12}. It has been suggested that after partial gastrectomy such features may be the result of abnormal proliferation by bacteria in the gastrointestinal tract (Bohmansson 1950, Naish & Capper 1953, Butler, Capper & Naish 1954, Adams 1958, Amendola 1965). Previous investigations have been on patients who had undergone partial gastrectomy and concerned mainly the possible relationship of abnormal bacterial proliferation to the occurrence of steatorrhoea (Goldstein, Wirts & Kramer 1961, Wirts & Goldstein 1963, Tabaqchali & Booth 1966). We too have studied the problem in an attempt to establish to what extent gastrointestinal contamination by a faecal type flora coincides with the occurrence of steatorrhoea, and also diarrhoea and impaired absorption of vitamin B_{12}, and to try to assess the importance or otherwise of gastric anacidity in enabling such contamination to occur. In view of the popularity of gastroenterostomy with vagotomy in the surgical management of patients with peptic ulceration, we have studied a group of patients who had undergone this procedure in addition to a number who had been treated by partial gastrectomy.

MATERIAL AND METHODS

There were twenty-six patients who had undergone partial gastrectomy with a Billroth II type anastomosis, and twenty-one who had undergone gastroenterostomy with vagotomy. The mean interval since operation for each group with standard deviation was 11.3 ± 3.6 years and 4.4 ± 1.7 years respectively. All subjects were ambulant throughout the study.

The technique of sampling juice from the stomach or stomach remnant and the jejunum and the method of estimating bacteria in this

quantitatively has already been fully described (Dellipiani & Girdwood 1964). In one patient it was not possible to obtain gastric juice. By the use of MacConkey agar and blood agar plates cultured aerobically and of blood agar plates and plates of Willis & Hobbs' medium (1959) cultured anaerobically, it was hoped to identify quantitatively organisms of the faecal type as represented by *Enterobacteriaceae*, *Streptococcus faecalis* and *Clostridium welchii*. In about half the patients with a partial gastrectomy *Bacteroides* were looked for using neomycin blood agar plates cultured anaerobically for up to five days (Smith & Crabb 1961). The characteristics of the individual colonies were examined by Gram staining and where necessary appropriate sugar reactions were carried out. Thus *Enterobacteriaceae* were subdivided into the groups proposed by the International Committee of Bacterial Nomenclature and Taxonomy (Enterobacteriaceae Subcommittee 1958). The finding of faecal type organisms as represented by the family *Enterobacteriaceae* or *Streptococcus faecalis* in a concentration of 10^4/ml or more in the anastomosed stomach or stomach remnant or the jejunum was considered to be abnormal. This figure is based on previously reported findings in the upper small intestine of normal controls (Dellipiani & Girdwood 1964).

Steatorrhoea was measured by estimating the fat content of a five-day stool specimen (van de Kamer et al. 1949). The serum vitamin B_{12} level was determined microbiologically using *Lactobacillus leichmanni* as the test organism on patients other than those on vitamin B_{12} therapy. Our normal range is 170-1000 $\mu\mu$g/ml. Vitamin B_{12} absorption was measured by the urinary excretion method of Schilling (1953), an output of less than 8.5 per cent of the administered dose in 24 hours being considered abnormal. The acid secretion of the stomach was measured following augmented histamine stimulation (Kay 1953, Card, Marks & Sircus 1955). No attempt was made to block off the gastroenterostomy stoma during this test.

The objective assessment of diarrhoea can be difficult. Diarrhoea was considered to be present when the patient regularly passed at least four bowel motions daily and had not done so prior to operation. Some of the patients suffered from intermittent diarrhoea.

Table I. *Stool fat related to gastrointestinal flora after gastric surgery*

	After gastroenterostomy		After partial gastrectomy	
	Nos. studied	Mean stool fat g per day with S.D.	Nos. studied	Mean stool fat g per day with S.D.
Gastric Flora				
Normal	15	6.9 ± 4.2	11	5.2 ± 2.5
Abnormal	4	6.2 ± 4	9	5.9 ± 2.5
	19		20	
Jejunal Flora				
Normal	11	5.8 ± 2.2	11	4.9 ± 2.3
Abnormal	8	8.1 ± 5.4	10	6.3 ± 2.7
	19		21	

Abnormal Flora = 10^4 viable organisms per ml aspirate and over.

RESULTS

Faecal type organisms were often isolated from the gastric and jejunal juices of these patients, the findings being similar regardless of the previous type of surgery.* In about one-third of the gastric aspirates and one-half of the jejunal aspirates of all patients the counts were classified as abnormal, ranging from 2×10^4 to 3×10^9 viable organisms per ml aspirate. Coliform type organisms were particularly common, the most prominent group being *Escherichia. Clostridium welchii* was found once only, in the jejunal juice of a patient with a partial gastrectomy. These findings are in contrast to those in normal controls where the stomach and small intestine are free from significant numbers of lower bowel organisms (Nichols & Glenn 1940, Cregan & Hayward 1953, Dellipiani & Girdwood 1964, Dellipiani & Shah 1967).

In Table I the mean daily fat excretion in the stools has been related to the finding of a normal

* The detailed findings on these patients willl be found on pages 164 and 165.

Table II. *Numbers of patients with complications after partial gastrectomy and relationships of these to gastrointestinal flora*

	Nos. studied	Diar-rhoea	Achlor-hydria	Impaired absorpt. of Vitamin B_{12}
Gastric Flora				
Normal	14	0	10*	2
Abnormal	11	1	9	8
	25			
Jejunal Flora				
Normal	14	0	9*	4
Abnormal	12	1	11	7
	26			

Abnormal flora = 10^4 viable organisms per ml and over.

*Gastric acidity in one patient with normal flora not determined.

Table III. *Numbers of patients with complications after gastroenterostomy and relationships of these to gastrointestinal flora*

	Nos. studied	Diar-rhoea	Achlor-hydria	Impaired absorpt. of Vitamin B_{12}
Gastric Flora				
Normal	17	4	4*	0
Abnormal	4	2	4	4
	21			
Jejunal Flora				
Normal	12	3	3*	0
Abnormal	9	3	5	4
	21			

Abnormal flora = 10^4 viable organisms per ml aspirate and over.

*Gastric acidity in two patients with normal flora not determined.

or abnormal faecal type flora in the stomach, stomach remnant, or jejunum of the patients. Presenting the results in this manner avoids the difficulty of having to define steatorrhoea and any bias which might result from this. It is seen that in both groups of patients fat excretion in the stools was higher when the flora was abnormal in the jejunum. The differences, however, are small and not statistically significant.

In Tables II and III the bacteriological findings in the two groups of patients have been related to the presence of diarrhoea, achlorhydria, and impaired absorption of vitamin B_{12}. Diarrhoea occurred in only one patient with a partial gastrectomy and in six patients after gastroenterostomy with vagotomy. Patients with intermittent diarrhoea have been included as cases of diarrhoea in the Table. There was no statistically significant relationship between the finding of diarrhoea and the occurrence of an abnormal flora in either the stomach or jejunum.

When the flora is studied in relation to gastric acidity it is seen that the great majority of patients with an abnormal flora in the stomach or jejunum after either operation had achlorhydria. Nevertheless it can also be seen that in a considerable number of patients with achlorhydria the flora was normal, this being particularly noticeable after partial gastrectomy.

Table IV. *Vitamin B_{12} absorption related to gastrointestinal flora in achlorhydric patients after gastric surgery*

	After gastro-enterostomy		After partial gastrectomy	
	Nos. studied	Nos. with impaired absorption of Vit. B_{12}	Nos. studied	Nos. with impaired absorption of Vit. B_{12}
Gastric Flora				
Normal	4	0	9	2
Abnormal	3	3	9	7
	7		18	
Jejunal Flora				
Normal	3	0	9	3
Abnormal	5	4	11	7
	8		20	

Abnormal flora = 10^4 viable organisms per ml aspirate and over.

Faecal type flora in stomach remnant and jejunum of twenty-six patients with partial gastrectomy

No.	Organisms found	Gastric aspirate	Jejunal aspirate	Years since op.	Serum Vit. B_{12} $\mu\mu$g/ml	Vitamin B_{12} absorption	Diarrhoea	Stool fat g/day	Acid Secretion mEq
1	Escherichia	Not done	1×10^5	16	62	Abnormal	No	10.4	0
	Cl. welchii	Not done	1×10^6						
2	Escherichia	3×10^4	2×10^6	16	546	Normal	Yes	5.4	2.8
3	Escherichia	–	9×10^5	7	589	Normal	No	Not done	0
4	Proteus	2×10^3	2×10^3	22	Not done	Abnormal	No	6.2	0
5	–	–	–	10	Not done	Abnormal	No	Not done	0
6	Escherichia	1×10^7	6×10^6	10	Not done	Abnormal	No	4.0	0
	Citrobacter	5×10^2	8×10^5						
7	Escherichia	2×10^4	3×10^3	11	395	Abnormal ×2	No	3.4	1.3
8	Escherichia	6×10^4	3×10^3	17	81	Abnormal	No	Not done	0
	Strep. faecalis	3×10^3	–						
9	Klebsiella	4×10^4	4×10^4	16	< 50	Abnormal	No	3.1	0
10	Escherichia	2×10^5	1×10^6	8	< 50	Abnormal	No	Not done	0
11	–	–	–	12	110	Normal	No	Not done	Not done
12	–	–	–	13	525	Normal	No	1.4	0
13	Escherichia	8×10^7	3×10^7	14	287	Normal	No	6.2	0
	Strep. faecalis	7×10^6	2×10^5						
14	Escherichia	4×10^5	2×10^5	11	152	Abnormal	No	8.3	0
	Klebsiella	2×10^5	–						
15	Proteus	–	9×10^6	10	985	Normal	No	6.5	0
16	–	–	–	15	190	Normal	No	2.5	1.6
17	–	–	–	9	452	Normal	No	5.7	4.1
18	–	–	–	9	350	Normal	No	5.0	2.8
19	–	–	–	9	281	Normal	No	10.9	0
20	–	–	–	9	201	Normal	No	2.3	0
21	–	–	–	9	365	Normal	No	6.7	0
22	Escherichia	2×10^5	5×10^5	8	Not done	Normal	No	6.4	0
23	Escherichia	2×10^4	4×10^7	9	Not done	Abnormal	No	10.3	0
24	Escherichia	5×10^6	5×10^5	8	98	Abnormal	No	2.1	0
25	–	–	–	8	129	Normal	No	5.2	0
26	–	–	–	8	546	Normal	No	4.6	0

Results expressed as numbers of viable organisms per ml aspirate.
– in columns, 2, 3 and 4 indicates organisms present in concentration of less than 250/ml aspirate.

So far as impaired absorption of vitamin B_{12} is concerned there would appear to be a significant relationship between impaired absorption and the presence of an abnormal flora particularly in the patients who had undergone a gastroenterostomy. This must be interpreted with caution in view of the effects of gastric acidity. Because of this the findings were analysed in the cases with achlorhydria to see if there was any relationship between the bacteriological findings and impaired absorption of vitamin B_{12}. The results of this analysis are shown in Table IV and are discussed further below.

DISCUSSION

Steatorrhoea. Goldstein and his colleagues considered that there was a relationship between abnormal bacterial proliferation, especially of

Faecal type flora in stomach and small intestine of twenty-one patients with gastroenterostomy and vagotomy

No.	Organisms found	Gastric aspirate	Jejunal aspirate	Years since op.	Serum Vitamin B_{12} $\mu\mu$g/ml	Vitamin B_{12} absorption	Diarrhoea	Stool fat g/day	Acid Secretion mEq
1	Escherichia	8×10^7	8×10^7	2	138	Abnormal	Yes	5.1	0
2	Escherichia	3×10^4	8×10^7	2	283	Abnormal	Yes	13	0
3	Citrobacter	–	2×10^4	4	366	Normal	No	3.7	2.8
4	–	–	–	3	452	Normal	Intermittent	0.8	Not done
5	Citrobacter	–	2×10^3	3	429	Normal	No	6.3	34.2
6	Escherichia Strep. faecalis	3×10^3 –	7×10^7 3×10^9	5	246	Normal	No	15	8.8
7	Escherichia Klebsiella	– –	5×10^6 5×10^6	1	375	Normal	Yes	3.4	0
8	Escherichia Strep. faecalis	6×10^7 2×10^7	8×10^7 9×10^7	5	492	Abnormal	No	3.5	0
9	–	–	–	4	622	Normal	No	8	0
10	–	–	–	4	939	Normal	No	4.1	2.6
11	–	–	–	4	502	Normal	No	6.5	19.5
12	–	–	–	7	924	Normal	No	8	0
13	–	–	–	7	843	Normal	No	Not done	Not done
14	Escherichia	–	5×10^5	7	513	Normal	No	Not done	4.9
15	–	–	–	6	457	Normal	No	4.9	2.5
16	–	–	–	2	1000	Normal	Yes	7	25.5
17	Escherichia	3×10^2	5×10^3	6	197	Normal	No	6.6	0
18	Escherichia	2×10^5	1×10^8	5	131	Abnormal	No	3.2	0
19	Escherichia Alk. Dispar	– –	2×10^6 1×10^6	5	333	Normal	No	17.6	1.0
20	–	–	–	6	678	Normal	Intermittent	8.2	8.4
21	–	–	–	5	373	Normal	No	3.4	10.9

Results expressed as numbers of viable organisms per ml aspirate.
– in columns 2, 3 and 4 indicates organisms present in concentration of less than 250/ml aspirate.

lower bowel type organisms, in the afferent loop in patients with a partial gastrectomy and the presence though not the degree of steatorrhoea (Goldstein et al. 1961, Wirts & Goldstein 1963). More recently Tabaqchali & Booth (1966) have reported their findings in similar patients and concluded that those with bacterial counts of more than 1×10^8 ml had more pronounced steatorrhoea.

Our own method of study assesses bacterial contamination in the gastrointestinal tract itself, whether this arises from the blind loop itself or by some other means, in patients who were *not fasting* throughout the period of the study (French 1961). Using our definition of normality, i.e. less than 10^4 viable organisms/ml, we have not found the daily fat excretion to differ significantly between those patients with normal and those with abnormal bacterial counts in the sites examined. This finding applies to all patients regardless of the type of operation. It could be that our definition of normality, though supported by other authors (Kalser et al. 1966), is either too strict or that bacterial counts must rise considerably above the normal before steatorrhoea occurs. Relating the stool fat content to counts of 10^5/ml and over,

10^6/ml and over, and 10^7/ml and over, however, does not demonstrate any statistically significant relationship.

The findings do not exclude the possibility that steatorrhoea can be caused by abnormal proliferation of faecal type organisms, though our results indicate that steatorrhoea can be absent even when the juices contain abnormal numbers of organisms. We have made similar observations in some patients with small intestinal diverticula. On the other hand, steatorrhoea occurred in some of the patients in the present study in the absence of abnormal bacteriological findings and it is possible that inadequate mixing of food with digestive enzymes or impaired pancreatic function such as has been suggested in postgastrectomy patients (Wollaeger 1950, Shingelton et al. 1957, Lundh 1958, Tyor & Ruffin 1958, Butler 1960) may be responsible for the abnormal fat loss in these cases. Another possibility is that some other organism is responsible for the steatorrhoea in these patients and strains of the genus *Bacteroides* have been suggested especially in view of their in vitro activity on bile salts (Drasar et al. 1966). *Bacteroides* is a difficult organism to isolate and we have looked for it in only half of our patients with a partial gastrectomy. They were found in two cases, the daily stool fat of these cases being 5.2 and 4.6 g.

Diarrhoea. The case of diarrhoea after gastric surgery is not understood and our findings show that diarrhoea and steatorrhoea can exist independently of each other in both groups of patients. This supports the observations of Clark, Crooks, Dawson & Mitchell (1964) in postgastrectomy patients. As usually occurs, the incidence of diarrhoea in our patients was higher after gastroenterostomy with vagotomy than after partial gastrectomy; however, the bacteriological findings in the two groups were similar. Though only one patient after partial gastrectomy had diarrhoea, half of the patients with this type of operation had an abnormal gastrointestinal flora. There is no support in the present findings for the suggestion, most recently voiced by Amendola (1965), that diarrhoea after gastric surgery may be related to abnormal bacterial proliferation.

Achlorhydria. It has for many years been thought that gastric acidity is important in keeping the small intestine free of bacterial contamination and the bacteriological findings of Davidson (1928) and more recently of Sherwood and his colleagues (1964) in the small intestine of patients with pernicious anaemia would seem to support this. It is obvious that a low pH will inhibit the growth of bacteria and this is reflected in the fact that in this study very few patients with free acid in the stomach ($pH < 3.5$) after either operation had an abnormal gastrointestinal flora and even in these cases the acid values were low. However, about half of the patients who developed achlorhydria ($pH > 6$) had normal bacteriological findings. This suggests that mechanisms other than gastric acidity operate to keep the intestine free from abnormal bacterial proliferation. This is in keeping with the work of Cregan, Dunlop & Hayward (1953) and Mainguet & Cattan (1960) and supports our own previous findings in patients with pernicious anaemia (Dellipiani & Girdwood 1964). At that time we thought that peristalsis might be important in the normal gastrointestinal tract by preventing any tendency for the intestinal contents to become stagnant. Certainly gastric surgery of the type being discussed not only creates conditions for stagnation but must render peristalsis less effective.

Vitamin B_{12} deficiency. Failure to absorb vitamin B_{12} may follow partial gastrectomy and gastroenterostomy. This is usually due to lack of intrinsic factor and only rarely to a blind loop (Adams 1958, Goldstein et al. 1961). Impaired absorption of the vitamin in our patients was commoner after partial gastrectomy but the interval after operation was longer in these patients. It could always be corrected by intrinsic factor. Except in one case in which there was a little acid, achlorhydria was present in the patients with impaired absorption of vitamin B_{12}. However, only half the patients in either group who had achlorhydria had impaired absorption of the vitamin, suggesting that loss of acid secretion usually precedes the loss of intrinsic factor as is usual also in pernicious anaemia.

The fact that impaired absorption of vitamin B_{12} was corrected by intrinsic factor in these cases makes it unlikely that micro-organisms could be playing any significant role in vitamin B_{12} absorption. Nonetheless by far the majority of patients with impaired absorption of vitamin B_{12} had an abnormal gastrointestinal flora particularly after gastroenterostomy. These figures might be influenced by the fact that free acid was commonly present in the stomachs of patients with normal absorption and, as discussed above, these patients will tend to have normal bacteriological findings. Because of this the findings were analysed in the patients with achlorhydria as is shown in Table IV. Only in patients after partial gastrectomy were the numbers large enough for analysis and no statistical relationship is demonstrable.

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